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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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PRIORITY DOCUMENT

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Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Blatt 2 der Bescheinigung
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Page 2 de l'attestation

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Method for detection of drug-selected mutations in the protease gene.

1. FIELD OF THE INVENTION

The present invention relates to the field of HIV diagnosis. More particularly, the present invention relates to the field of diagnosing the susceptibility of an HIV sample to antiviral drugs used to treat HIV infection.

The present invention relates to a method for the rapid and reliable detection of drug-selected mutations in the HIV protease gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridisation assay.

2. BACKGROUND OF THE INVENTION

The human immunodeficiency virus (HIV) is the ethiological agent for the acquired immunodeficiency syndrome (AIDS). HIV, like other retroviruses, encodes an aspartic protease that mediates the maturation of the newly produced viral particle by cleaving viral polypeptides into their functional forms (Hunter *et al*). The HIV protease is a dimeric molecule consisting of two identical subunits each contributing a catalytic aspartic residue (Navia *et al*, Whodawer *et al*, Meek *et al*). Inhibition of this enzyme gives rise to noninfectious viral particles that cannot establish new cycles of viral replication (Kohl *et al*, Peng *et al*).

Attempts to develop inhibitors of HIV-1 protease were initially based on designing peptide compounds that mimicked the natural substrate. The availability of the 3-dimensional structure of the enzyme have more recently allowed the rational design of protease inhibitors (PI) using computer modelling (Huff *et al*, Whodawer *et al*). A number of second generation PI that are partially peptidic or entirely nonpeptidic have proven to exhibit particularly potent antiviral effects in cell culture. Combinations of various protease inhibitors with nucleoside and non-nucleoside RT inhibitors have also been studied extensively *in vitro*. In every instance, the

combinations have been at least additive and usually synergistic.

In spite of the antiviral potency of many recently developed HIV-1 PI, the emergence of virus variants with decreased sensitivity to these compounds has been described both in cell culture and in treated patients thereby escaping the inhibitory effect of the antiviral (Condra *et al.*). Emergence of resistant variants depends on the selective pressure applied to the viral population. In the case of a relatively ineffective drug, selective pressure is low because replication of both wild-type virus and any variants can continue. If a more effective drug suppresses replication of virus except for a resistant variant, then that variant will be selected. Virus variants that arise from selection by PI carry several distinct mutations in the protease coding sequence that appear to emerge sequentially. A number of these cluster near the active site of the enzyme while others are found at distant sites. This suggests conformational adaptation to primary changes in the active site and in this respect certain mutations that increase resistance to PI also decrease protease activity and virus replication.

Amongst the PI, the antiviral activity of the PI ritonavir (A-75925; ABT-538), nelfinavir (AG-1343), indinavir (MK-639; L735; L524) and saquinavir (Ro 31-8959) have been approved by the Food and Drug Administration and are currently under evaluation in clinical trials involving HIV-infected patients. The VX-487 (141W94) antiviral compound is not yet approved. The most important mutations selected for the above compounds and leading to gradually increasing resistance are found at amino acid (aa) positions 30 (D to N), 46 (M to I), 48 (G to V), 50 (I to V), 54 (I to A, I to V), 82 (V to A, or F, or I, or T), 84 (I to V) and 90 (L to M). Other mutations associated with drug resistance to the mentioned compounds have been described (Schinazi *et al*). Saquinavir-resistant variants, which usually carry mutations at amino acid positions 90 and/or 48, emerge in approximately 45% of patients after 1 year of monotherapy. Resistance appears to develop less frequently with higher doses of saquinavir. Resistance to indinavir and ritonavir requires multiple mutations, usually at greater than 3 and up to 11 sites, with more amino acid substitutions conferring higher levels of resistance. Resistant isolates usually carry mutations at codons 82, 84, or 90. In the case of ritonavir, the mutation at codon 82 appears first in most patients. Although mutant virions resistant to saquinavir are not cross-resistant to indinavir or ritonavir, isolates resistant to indinavir are generally ritonavir resistant and visa versa. Resistance to either indinavir or ritonavir usually results in cross-resistance to saquinavir. Approximately one third of indinavir resistant isolates are cross-resistant to nelfinavir

as well.

The regime for an efficient antiviral treatment is currently not clear at all. Patterns of reduced susceptibility to HIV protease inhibitors have been investigated *in vitro* by cultivating virus in the presence of PI. These data, however, do not completely predict the pattern of amino-acid changes actually seen in patients receiving PI. Knowledge of the resistance and cross-resistance patterns should facilitate selection of optimal drug combinations and selection of sequences with non-overlapping resistance patterns. This would delay the emergence of cross-resistant viral strains and prolong the duration of effective antiretroviral activity in patients. Therefore, there is need for methods and systems which detect these mutational events in order to give a better insight into the mechanisms of HIV resistance. Further, there is need for methods and systems which can provide data important for the antiviral therapy to follow in a more time-efficient and economical manner compared to the conventional cell-culture selection techniques.

3. AIMS OF THE INVENTION

It is an aim of the present invention to develop a rapid and reliable detection method for determination of the antiviral drug resistance of viruses which contain protease genes such as HIV retroviruses present in a biological sample.

More particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV protease gene wild type and mutation codons involved in the antiviral resistance in one single experiment.

It is also an aim of the present invention to provide an HIV protease genotyping assay or method which allows to infer the nucleotide sequence at codons of interest and/or the amino acids at the codons of interest and/or the antiviral drug selected spectrum, and possibly also infer the HIV type or subtype isolate involved.

Even more particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV protease gene polymorphisms representing wild-type and mutation codons in one single experimental setup.

It is another aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated or polymorphic HIV protease sequences

conferring resistance to one or more antiviral drugs, such as ritonavir (A-75925; ABT-538), nelfinavir (AG-1343), indinavir (MK-639; L735; L524), saquinavir (Ro 31-8959) and VX-478 (141W94) or others (Shinazi *et al*).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated or polymorphic HIV protease sequences conferring resistance to ritonavir (A-75925; ABT-538).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to nelfinavir (AG-1343).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to indinavir (MK-639; L735; L524).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to saquinavir (Ro 31-8959).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to VX-478 (141W94).

It is also an aim of the present invention to select particular probes able to determine and/or infer cross-resistance to HIV protease inhibitors.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease from mutated HIV protease sequences involving at least one of amino acid positions 30 (D to N), 46 (M to I), 48 (G to V), 50 (I to V), 54 (I to A or V), 82 (V to A or F or I or T), 84(I to V) and 90 (L to M) of the viral protease gene.

It is particularly an aim of the present invention to select a particular set of probes, able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to any of the antiviral drugs defined above with this particular set of probes being used in a reverse hybridisation assay.

It is moreover an aim of the present invention to combine a set of selected probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to antiviral drugs with another set of selected probes able to identify the HIV isolate,

type or subtype present in the biological sample, whereby all probes can be used under the same hybridisation and wash-conditions.

It is also an aim of the present invention to select primers enabling the amplification of the gene fragment(s) determining the antiviral drug resistance trait of interest.

It is also an aim of the present invention to select particular probes able to identify mutated HIV protease sequences resulting in cross-resistance to antiviral drugs.

The present invention also aims at diagnostic kits comprising said probes useful for developing such a genotyping assay.

The present invention also aims at diagnostic kits comprising said primers useful for developing such a genotyping assay.

4. DETAILED DESCRIPTION OF THE INVENTION.

All the aims of the present invention have been met by the following specific embodiments.

According to one embodiment, the present invention relates to a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising:

a) if need be, releasing, isolating or concentrating the polynucleic acids present in the sample;

b) if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair;

c) hybridizing the polynucleic acids of step a) or b) with at least one of the following probes:

probes specifically hybridizing to a target sequence comprising codon 30;

probes specifically hybridizing to a target sequence comprising codon 46 and/or 48;

probes specifically hybridizing to a target sequence comprising codon 50;

probes specifically hybridizing to a target sequence comprising codon 54;

probes specifically hybridizing to a target sequence comprising codon 82 and/or 84;

probes specifically hybridizing to a target sequence comprising codon 90;

or the complement of said probes,

further characterized in that said probes specifically hybridize to any of the target sequences presented in figure 1, or to the complement of said target sequences;

d) inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.

The numbering of HIV-1 protease gene encoded amino acids is as generally accepted in literature. Mutations that give rise to an amino acid change at position 48 or 90 are known to confer resistance to saquinavir (Erlebe *et al;* Tisdale *et al*). An amino acid change at codon 46 or 54 or 82 or 84 results in ritonavir and indinavir resistance (Kempf *et al*; Emini *et al*; Condra *et al*). Amino acid changes at positions 30 and 46 confer resistance to nelfinavir (Patick *et al*) and amino acid changes at position 50 confers resistance to VX-487 (Rao *et al*). Therefore, the method described above allows to determine whether a HIV strain is susceptible or resistant to any of the drugs mentioned above. This method can be used, for instance, to screen for mutations conferring resistance to any of the mentioned drugs before initiating therapy. This method may also be used to screen for mutations that may arise during the course of therapy (i.e. monitoring of drug therapy). It is obvious that this method may also be used to determine resistance to drugs other than the above-mentioned drugs, provided that resistance to these other drugs is linked to mutations that can be detected by use of this method. This method may also be used for the specific detection of polymorphic nucleotides. It is to be understood that the said probes may only partly overlap with the targets sequences of figure 1, table 2 and table 3, as long as they allow for specific detection of the relevant polymorphic nucleotides as indicated above. The sequences of figure 1, table 2 and table 3 were derived from polynucleic acid fragments comprising the protease gene. These fragments were obtained by PCR amplification and were inserted into a cloning vector and sequence analysed as described in example 1. It is to be noted that some polynucleic acid fragments comprised polymorphic nucleotides in their sequences which have not been previously disclosed. These novel polymorphic nucleotide sequences are represented in table 4 below.

TABLE 4: Polymorphic nucleotide sequences.

51	52	53	54	55	56	57	58	codon position
gga	ggt	ttt	atc	aaa	gta	aga	cag	consensus sequence
GGA	GGT	TTT	ATC	AAA	GTC	AGA	CAA	SEQ ID NO 478
GGA	GGT	TTC	ATT	AAG	GTA	AAA	CAG	SEQ ID NO 479
GGA	GGT	TTT	ATT	AAG	GTA	AGA	CAG	SEQ ID NO 480
GGA	GGT	TTT	ATT	AAA	GTA	AGA	CAA	SEQ ID NO 481

GGA GGC TTT ATC AAA GTA AGA CAA	SEQ ID N0 482
GGA GGT TTT ATC AAA GTC AGA CAA	SEQ ID N0 483

78 79 80 81 82 83 84 85	**codon position**
gga cct aca cct gtc aac ata att gg	**consensus sequence**
GGA CCT ACA CCG GTC AAC ATA ATT GG	SEQ ID N0 484
GGA CCT ACA CCT GCC AAT ATA ATT GG	SEQ ID N0 485
GGA CCT ACG CCC TTC AAC ATA ATT GG	SEQ ID N0 486
GGA CCG ACA CCT GTC ACC ATA ATT GG	SEQ ID N0 487
GGA CCT ATA CCT GTC AAC ATA ATT GG	SEQ ID N0 488

87 88 89 90 91 92 93 94	**codon position**
a aga aat ctg ttg act cag att ggc	**consensus sequence**
A AAA AAT CTG ATG ACT CAG ATT GGC	SEQ ID N0 489
A AGA ACT CTG TTG ACT CAG CTT GGA	SEQ ID N0 490
A AGA AAT ATG ATG ACC CAG CTT GGC	SEQ ID N0 491
A AGA AAT ATA ATG ACT CAG CTT GGA	SEQ ID N0 492
A AGA AAT CTG CTG ACT CAG ATT GGG	SEQ ID N0 493
A AGA AAT CTG TTG ACA CAG CTT GGC	SEQ ID N0 494
A AGA AAT ATG TTG ACT CAG CTT GGT	SEQ ID N0 495
A AGA AAT TTG TTG ACT CAG ATT GGG	SEQ ID N0 496
A AGA AAT ATG TTG ACT CAG CTT GGT	SEQ ID N0 497
A AGA AAT ATG TTG ACT CAG CTT GGA	SEQ ID N0 498
A AGA AAT CTG TTG ACT CAG CTT GGA	SEQ ID N0 499
A AGA AAC CTG TTG ACT CAA CTT GGT	SEQ ID N0 500

The present invention thus also relates to these novel sequences, or a fragment thereof, wherein said fragment consists of at least 10, preferably 15, even more preferably 20 contiguous nucleotides and contains at least one polymorphic nucleotide. It is furthermore to be understood that these new polymorphic nucleotides may also be expected to arise in another sequence context than in the mentioned sequences. For instance a G at the third position of codon 55 is shown in SEQ ID N° 478 in combination with a T at the third position of codon 54, but a G at the third position of codon 55 may also be expected to occur in the context of a wild type sequence. It is

also to be understood that the above mentioned specifications apply to the complement of the said target sequences as well. This applies also to Figure 1.

According to a preferred embodiment the present invention relates to a method as indicated above, further characterized in that said probes are capable of simultaneously hybridizing to their respective target regions under appropriate hybridization and wash conditions allowing the detection of the hybrids formed.

According to a preferred embodiment, step c is performed using a set of at least 2, preferably at least 3, more preferably at least 4 and most preferably at least 5 probes meticulously designed as such that they show the desired hybridization results. In general this method may be used for any purpose that relies on the presence or absence of mutations that can be detected by this method, e.g. for genotyping.

The probes of table 1 have been optimized to give specific hybridization results when used in a LiPA assay (see below), as described in examples 2 and 3. These probes have thus also been optimized to simultaneously hybridize to their respective target regions under the same hybridization and wash conditions allowing the detection of hybrids. The sets of probes for each of the codons 30, 46/48, 50, 54 and 82/84 have been tested experimentally as described in examples 2 and 3. The reactivity of the sets shown in table 1 with 856 serum samples from various geographic origins was evaluated. It was found that the sets of probes for codons 30, 46/48, 50, 54 and 82/84 reacted with 98.9%, 99.6%, 98.5%, 99.2%, 95.4% and 97.2% of the test samples, respectively. The present invention thus also relates to the sets of probes for codons 30, 46/48, 50, 54, 82/84 and 90, shown in table 1.

According to another even more preferred embodiment, the present invention relates to a method as defined above, further characterized in that:

step b) comprises amplifying a fragment of the protease gene with at least one 5'-primer specifically hybridizing to a target sequence located between nucleotide position 210 and nucleotide position 260 (codon 87), more preferably between nucleotide position 220 and nucleotide position 260 (codon 87), more preferably between nucleotide position 230 and nucleotide position 260 (codon 87), even more preferably at nucleotide position 241 to nucleotide position 260 (codon 87) in combination with at least one suitable 3'-primer, and

step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the

probes specifically hybridizing to a target sequence comprising codon 90.

According to another even more preferred embodiment, the present invention relates to a method as defined above, further characterized in that:

step b) comprises amplifying a fragment of the protease gene with at least one 3'-primer specifically hybridizing to a target sequence located between nucleotide position 253 (codon 85) and nucleotide position 300, more preferably between nucleotide position 253 (codon 85) and nucleotide position 290, more preferably between nucleotide position 253 (codon 85) and nucleotide position 280, even more preferably at nucleotide position 253 (codon 85) to nucleotide position 273 (codon 91), in combination with at least one suitable 5'-primer, and step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence comprising any of codons 30, 46, 48, 50, 52, 54, 82 and 84.

It has been found, unexpectedly, that an amplified nucleic acid fragment comprising all of the above-mentioned codons, does not hybridize optimally to probes comprising codon 82, 84 or 90 On the other hand, a shorter fragment, for instance the fragment which is amplified by use of the primers Prot41bio and Prot6bio with respectively seq id no 5 and seq id no 4, hybridizes better to probes comprising codon 90. The present invention thus also relates to a method as defined above, further characterized in that the 5'-primer is seq id no 5 and the 3'-primer is seq id no 4. Likewise, another shorter fragment, for instance the fragment which is amplified by use of the primers Prot2bio and Prot31bio with respectively seq id no 3 and seq id no 6 , was found to hybridize better to probes comprising codon 82 and/or 84. Hence the present invention also relates to a method as defined above, further characterized in that the 5'-primer is seq id no 5 and the 3'-primer is seq id no 4.

Different techniques can be applied to perform the sequence-specific hybridization methods of the present invention. These techniques may comprise immobilizing the amplified HIV polynucleic acids on a solid support and performing hybridization with labelled oligonucleotide probes. HIV polynucleic acids may also be immobilized on a solid support without prior amplification and subjected to hybridization. Alternatively, the probes may be immobilized on a solid support and hybridization may be performed with labelled HIV polynucleic acids, preferably after amplification. This technique is called reverse hybridization. A convenient reverse hybridization technique is the line probe assay (LiPA). This assay uses oligonucleotide probes immobilized as

parallel lines on a solid support strip (Stuyver et al., 1993). It is to be understood that any other technique based on the above-mentioned methods is also covered by the present invention.

According to another preferred embodiment, the present invention relates to any of the probes mentioned above and/or to any of the primers mentioned above, with said primers and probes being designed for use in a method for determining the susceptibility to antiviral drugs of HIV viruses in a sample. According to an even more preferred embodiment, the present invention relates to the probes with seq id no 7 to seq id no 477, more preferably to the seq id no Mentioned in Table 1 and to the primers with seq id no 3, 4, 5 and 6. The skilled man will recognize that the said probes and primers may be adapted by addition or deletion of one or more nucleotides at their extremities. Such adaptations may be required if the conditions of amplification or hybridization are changed, or if the amplified material is RNA instead of DNA, as is the case in the NASBA system.

According to another preferred embodiment, the present invention relates to a diagnostic kit enabling a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said kit comprising:

a) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in said sample;

b) when appropriate, at least one of the primers of any of claims 4 to 6;

c) at least one of the probes of any of claims 1 to 3, possibly fixed to a solid support;

d) a hybridization buffer, or components necessary for producing said buffer;

e) a wash solution, or components necessary for producing said solution;

f) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

h) when appropriate, a means for attaching said probe to a solid support.

DEFINITIONS

The following definitions serve to illustrate the terms and expressions used in the present invention.

The term "antiviral drugs" refers particularly to any antiviral protease inhibitor. Examples of such antiviral drugs and the mutation they may cause in the HIV protease gene are disclosed in Schinazi et al., 1997. The contents of the latter two documents particularly are to be considered as forming part of the present invention. The most important antiviral drugs focussed at in the present invention are disclosed in Tables 1 to 2.

The target material in the samples to be analysed may either be DNA or RNA, e.g.: genomic DNA, messenger RNA, viral RNA or amplified versions thereof. These molecules are also termed polynucleic acids.

It is possible to use genomic DNA or RNA molecules from HIV samples in the methods according to the present invention.

Well-known extraction and purification procedures are available for the isolation of RNA or DNA from a sample (f.i. in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press (1989)).

The term "probe" refers to single stranded sequence-specific oligonucleotides which have a sequence which is complementary to the target sequence to be detected.

The term "target sequence" as referred to in the present invention describes the wild type nucleotide sequence, or the sequence comprising one or more polymorphic nucleotides of the protease gene to be specifically detected by a probe according to the present invention. This nucleotide sequence may encompass one or several nucleotide changes. Target sequences may refer to single nucleotide positions, codon positions, nucleotides encoding amino acids or to sequences spanning any of the foregoing nucleotide positions. In the present invention said target sequence often includes one or two variable nucleotide positions.

The term "polymorphic nucleotide" indicates a nucleotide in the protease gene of a particular HIV virus that is different from the nucleotide at the corresponding position in at least one other HIV virus. The polymorphic nucleotide may or may not give rise to resistance to an antiviral drug.

It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases. The target sequences as defined in the present invention provide

sequences which should be complementary to the central part of the probe which is designed to hybridize specifically to said target region.

The term "complementary" as used herein means that the sequence of the single stranded probe is exactly the (inverse) complement of the sequence of the single-stranded target, with the target being defined as the sequence where the mutation to be detected is located.

"Specific hybridization" of a probe to a target sequence of the HIV polynucleic acids means that said probe forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions said probe does not form a duplex with other regions of the polynucleic acids present in the sample to be analysed.

Since the current application requires the detection of single basepair mismatches, very stringent conditions for hybridization are required, allowing in principle only hybridization of exactly complementary sequences. However, variations are possible in the length of the probes (see below), and it should be noted that, since the central part of the probe is essential for its hybridization characteristics, possible deviations of the probe sequence versus the target sequence may be allowable towards head and tail of the probe, when longer probe sequences are used. These variations, which may be conceived from the common knowledge in the art, should however always be evaluated experimentally, in order to check if they result in equivalent hybridization characteristics than the exactly complementary probes.

Preferably, the probes of the invention are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. Particularly preferred lengths of probes include 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups which do not essentially alter their hybridisation characteristics.

Probe sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

The probes according to the invention can be prepared by cloning of recombinant plasmids containing inserts including the corresponding nucleotide sequences, if need be by cleaving the latter out from the cloned plasmids upon using the adequate nucleases and recovering them, e.g.

by fractionation according to molecular weight. The probes according to the present invention can also be synthesized chemically, for instance by the conventional phospho-triester method.

The term "solid support" can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the background level of hybridization remains low. Usually the solid substrate will be a microtiter plate, a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead) or a chip. Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH_2 groups, SH groups, carboxylic groups, or coupling with biotin, haptens or proteins.

The term "labelled" refers to the use of labelled nucleic acids. Labelling may be carried out by the use of labelled nucleotides incorporated during the polymerase step of the amplification such as illustrated by Saiki et al. (1988) or Bej et al. (1990) or labelled primers, or by any other method known to the person skilled in the art. The nature of the label may be isotopic (^{32}P, ^{35}S, etc.) or non-isotopic (biotin, digoxigenin, etc.).

The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow to prime the synthesis of the extension products. Preferably the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of the required DNA or RNA targets, as well as on the conditions of primer use such as temperature and ionic strength.

The fact that amplification primers do not have to match exactly with the corresponding template sequence to warrant proper amplification is amply documented in the literature (Kwok et al., 1990).

The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS; Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990) or amplification by means of Qß replicase (Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

The oligonucleotides used as primers or probes may also comprise nucleotide analogues such as phosphorothiates (Matsukura et al., 1987), alkylphosphorothiates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may contain intercalating agents (Asseline et al., 1984).

As most other variations or modifications introduced into the original DNA sequences of the invention these variations will necessitate adaptions with respect to the conditions under which the oligonucleotide should be used to obtain the required specificity and sensitivity. However the eventual results of hybridisation will be essentially the same as those obtained with the unmodified oligonucleotides.

The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics, reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules, etc.

The "sample" may be any biological material taken either directly from the infected human being (or animal), or after culturing (enrichment). Biological material may be e.g. expectorations of any kind, broncheolavages, blood, skin tissue, biopsies, sperm, lymphocyte blood culture material, colonies, liquid cultures, faecal samples, urine etc.

The sets of probes of the present invention will include at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more probes. Said probes may be applied in two or more distinct and known positions on a solid substrate. Often it is preferable to apply two or more probes together in one and the same position of said solid support.

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied.

Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. The importance and effect of various assay conditions, explained further herein, are known to those skilled in the art.

The stability of the [probe : target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences, by terminating the hybrids with G:C base pairs, and by designing the probe with an appropriate Tm.

The beginning and end points of the probe should be chosen so that the length and %GC result in a Tm about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

Conditions such as ionic strenght and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that hybridization will increase as the ionic strenght of the reaction mixture increases, and that the thermal stability of the hybrids will increase with increasing ionic strenght. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohols, which disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the Tm. In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

It is desirable to have probes which hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid. The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the nontarget nucleic acid. In the present case, single base pair changes need to be detected, which requires conditions of very high stringency.

The length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. In some cases, there may be several sequences from a particular region, varying in location and length, which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another which differs merely by a single base. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly complementary base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition

may be used, preferred oligonucleotide probes of this invention are between about 5 to 50 (more particularely 10-25) bases in length and have a sufficient stretch in the sequence which is perfectly complementary to the target nucleic acid sequence.

Regions in the target DNA or RNA which are known to form strong internal structures inhibitory to hybridization are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of complementary nucleic acids to form a hydrogen bonded double strand. It is implicit that if one of the two strands is wholly or partially involved in a hybrid that it will be less able to participate in formation of a new hybrid. There can be intramolecular and intermolecular hybrids formed within the molecules of one type of probe if there is sufficient self complementarity. Such structures can be avoided through careful probe design. By designing a probe so that a substantial portion of the sequence of interest is single stranded, the rate and extent of hybridization may be greatly increased. Computer programs are available to search for this type of interaction. However, in certain instances, it may not be possible to avoid this type of interaction.

Standard hybridization and wash conditions are disclosed in the Materials & Methods section of the Examples. Other conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C.

Other solutions (SSPE (Sodium saline phosphate EDTA), TMACl (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. If need be, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances.

Primers may be labelled with a label of choice (e.g. biotin). Different primer-based target amplification systems may be used, and preferably PCR-amplification, as set out in the examples. Single-round or nested PCR may be used.

The term "hybridization buffer" means a buffer enabling a hybridization reaction to occur between the probes and the polynucleic acids present in the sample, or the amplified products, under the appropriate stringency conditions.

The term "wash solution" means a solution enabling washing of the hybrids formed under the appropriate stringency conditions.

The following examples only serve to illustrate the present invention. These examples are in no way intended to limit the scope of the present invention.

FIGURE AND TABLE LEGENDS

Figure 1: Natural and drug selected variability in the vicinity of codons 30, 46, 48, 50, 54, 82, 84, and 90 of the HIV-1 protease gene. The most frequently observed wild-type sequence is shown in the top line. Naturally occurring variations are indicated below and occur independently from each other. Drug-selected variants are indicated in bold

Figure 2 A: Reactivities of the selected probes for codon 30 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes are shown at the left and is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. At the bottom the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 B: Reactivities of the selected probes for codons 46 and 48 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 C: Reactivities of the selected probes for codon 50 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is

incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 D: Reactivities of the selected probes for codon 54 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 E.:Reactivities of the selected probes for codons 82 and 84 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 2 F: Reactivities of the selected probes for codon 90 immobilized on LiPA strips with reference material. The position of each probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. *: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived

from the probe reactivity, is indicated.

Figure 3: Sequence and position of the HIV-1 protease amplification primers. To obtain the reactivity with probes selected to determine the susceptibility to antiviral drugs involving codons 30, 46, 48, 50, 54, 82, and 84, nested amplification primers prot2bio(5' primer) and Prot31bio (3' primer) were designed. To obtain the reactivity with probes selected to determine the susceptibility to antiviral drugs involving codon 90, nested amplification primers Prot41bio (5' primer) and Prot6bio (3' primer) were designed.

Figure 4 A: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 30 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 4 B: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon s 46/48 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 4 C: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 50 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 4 D: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 54 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in

Table 1.

Figure 4 E: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codons 82/84 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 4 F: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 90 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

Figure 5 A: Geographical origin of 856 samples and reactivities with the different probes at codon position 30. The probes are indicated at the bottom.

Figure 5 B: Geographical origin of 856 samples and reactivities with the different probes at codon positions 46/48. The probes are indicated at the bottom.

Figure 5 C: Geographical origin of 856 samples and reactivities with the different probes at codon position 50. The probes are indicated at the bottom.

Figure 5 D: Geographical origin of 856 samples and reactivities with the different probes at codon position 54. The probes are indicated at the bottom.

Figure 5 E: Geographical origin of 856 samples and reactivities with the different probes at codon positions 82/84 . The probes are indicated at the bottom.

Figure 5 F: Geographical origin of 856 samples and reactivities with the different probes at codon position 90. The probes are indicated at the bottom.

Table 1: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as applied on the HIV-1 protease LiPA strip. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence.

Table 3: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as synthesised, immobilized and tested on LiPA strips. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence. The probes retained are indicated in table 1.

EXAMPLES

Example 1:

Selection of the plasma samples, PCR amplification and cloning of the PCR products.

Plasma samples (n=557) were taken from HIV type-1 infected patients and stored at -20°C until use. Plasma samples were obtained from naive and drug-treated patients. The drugs involved ritonavir, indinavir and saquinavir. The serum samples were collected from patients residing in Europe (Belgium, Luxemburg, France, Spain and UK), USA and Brazil.

HIV RNA was prepared from these samples using the guanidinium-phenol procedure. Fifty µl plasma was mixed with 150 µl Trizol®LS Reagent (Life Technologies, Gent, Belgium) at room temperature (volume ratio: 1unit sample/ 3 units Trizol). Lysis and denaturation occurred by carefully pipetting up and down several times, followed by an incubation step at room temperature for at least 5 minutes. Fourthy µl $CHCl_3$ was added and the mixture was shaken vigorously by hand for at least 15 seconds, and incubated for 15 minutes at room temperature. The samples were centrifuged at maximum 12,000g for 15 minutes at 4°C, and the colourless aqueous phase was collected and mixed with 100 µl isopropanol. To visualize the minute amounts of viral RNA, 20 µl of 1µg/µl Dextran T500 (Pharmacia) was added, mixed and left at room

temperature for 10 minutes. Following centrifugation at max. 12,000g for 10 minutes at 4°C and aspiration of the supernatant, the RNA pellet was washed with 200 µl ethanol, mixed by vortexing and collected by centrifugation at 7,500g for 5 minutes at 4°C. Finally the RNA pellet was briefly air-dried and stored at -20°C.

For cDNA synthesis and PCR amplification, the RNA pellet was dissolved in 15 µl random primers (20 ng/µl, pdN_6, Pharmacia), prepared in DEPC-treated or HPLC grade water. After denaturation at 70°C for 10 minutes, 5 µl cDNA mix was added, composed of 4 µl 5x AMV-RT buffer (250mM Tris.HCl pH 8.5, 100mM KCl, 30mM $MgCl_2$, 25 mM DTT), 0.4 µL 25mM dXTPs, 0.2 µl or 25U Ribonuclease Inhibitor (HPRI, Amersham), and 0.3 µl or 8U AMV-RT (Stratagene). cDNA synthesis occurred during the 90 minutes incubation at 42°C. The HIV -1 protease gene was than amplified using the following reaction mixture: 5 µl cDNA, 4.5 µl 10x Taq buffer, 0.3 µl 25 mM dXTPs, 1 µl (10 pmol) of each PCR primer, 38 µl H_2O, and 0.2 µl (1 U) Taq.

Codon positions involving resistance to saquinavir, ritonavir, indinavir, nelfinavir and VX-478 have been described (Shinazi *et al*) and PCR amplification primers were chosen outside these regions. The primer design was based on HIV-1 published sequences (mainly genotype B clade) (Myers *et al.*) and located in regions that showed a high degree of nucleotide conservation between the different HIV-1 clades. The final amplified region covered the HIV-1 protease gene from codon 9 to codon 99. The primers for amplification had the following sequence: outer sense primer Pr16: 5' bio-CAGAGCCAACAGCCCCACCAG3' (SEQ ID NO 1); nested sense primer Prot 2 bio: 5' CCT CAR ATC ACT CTT TGG CAA CG 3' (SEQ ID NO 3) ; nested antisense primer Prot 6 bio: 3' TAA TCR GGA TAA CTY TGA CAT GGT C 5' (SEQ ID NO 4); and outer antisense primer RT12: 5' bioATCAGGATGGAGTTCATAACCCATCCA3' (SEQ ID NO 2). Annealing occurred at 57°C, extension at 72°C and denaturation at 94°C. Each step of the cycle took 1 minute, the outer PCR contained 40 cycles, the nested round 35. Nested round PCR products were analysed on agarose gel and only clearly visible amplification products were used in the LiPA procedure. Quantification of viral RNA was obtained with the HIV Monitor™test (Roche, Brussels, Belgium).

Selected PCR products were cloned into the pretreated EcoRV site of the pGEMT vector (Promega). Recombinant clones were selected after α-complementation and restriction fragment length analysis, and sequenced using standard sequencing techniques with plasmid primers and

internal HIV protease primers. Sometimes biotinylated fragments were directly sequenced with a dye-terminator protocol (Applied Biosystems) using the amplification primers. Alternatively, nested PCR was carried out with analogs of the nested primers, in which the biotin group was replaced with the T7- and SP6-primer sequence, respectively. These amplicons were than sequenced with an SP6- and T7-dye-primer procedure.

Example 2:

Selection of a reference panel

Codon positions involving resistance to saquinavir, ritonavir, indinavir, nelfinavir and VX-478 have been described (Shinazi *et al. 1997*). It was the aim to clone in plasmids those viral protease genes that are covering the different genetic motifs at those important codon positions conferring resistance against the described protease inhibitors.

After careful analysis of 312 protease gene sequences, obtained after direct sequencing of PCR fragments, a selection of 47 PCR fragments which covered the different target polymorphisms and mutations were retained and cloned in plasmids using described cloning techniques. The selection of samples originated from naive or drug-treated European, Brazilian or US patients. These 47 recombinant plasmids are used as a reference panel, a panel which was sequenced on both strands, and biotinylated PCR products from this panel were used to optimize probes for specificity and sensitivity.

Although this panel of 47 samples is a representative selection of clones at this moment, it is important to mention here that this selection is an fact only a temporally picture of the variability of the virus, and a continuous update of this panel will be mandatory. This includes on ongoing screening for the new variants of the virus, and recombinant cloning of these new motifs.

Probe selection and LiPA testing.

To cover all the different genetic motifs in the reference panel, a total of 471 probes were designed (codon 30: 40 probes; codon 46/48: 72 probes; codon 50:55 probes; codon 54: 54 probes; codon 82/84: 130 probes; codon 90: 120 probes).Table 3 shows the different probes that were selected for the different codon positions.

It was the aim to adapt all probes to react specifically under the same hybridization and

wash conditions by carefully considering the % (G+C), the probe length, the final concentration of the buffer components, and hybridization temperature (Stuyver et al., 1997). Therefore, probes were provided enzymatically with a poly-T-tail using the TdT (Pharmacia) in a standard reaction condition, and purified via precipitation. For a limited number of probes with 3' T-ending sequences, an additional G was incorporated between the probe sequence and the poly-T-tail in order to limit the hybridising part to the specific probe sequence and to exclude hybridisation with the tail sequence. Probe pellets were dissolved in standard saline citrate (SSC) buffer and applied as horizontal parallel lines on a membrane strip. Control lines for amplification (probe 5' TAGGGGGAATTGGAGGTTTTAG 3 ', HIV protease aa 47 to aa 54) and conjugate incubation (biotinylated DNA) were applied alongside. Probes were immobilized onto membranes by baking, and the membranes were sliced into 4mm strips also called LiPA strips.

Selection of the amplification primers and PCR amplification was as described in example 1. In order to select specific reacting probes out of the 471 candidate probes, LiPA teste were performed with biotinylated PCR fragments from the reference panel. To perform LiPA tests, equal amounts (10 µl) of biotinylated amplification products and denaturation mixture (0.4 N NaOH/0.1% SDS) were mixed, followed by an incubation at room temperature for 5 minutes. Following this denaturation step, 2 ml hybridization buffer (2xSSC, 0.1% SDS, 50mM Tris pH7.5) was added together with a membrane strip and hybridization was carried out at 39°C for 30 min. Then, the hybridization mixture was replaced by stringent washing buffer (same composition as hybridisation buffer), and stringent washing occurred first at room temperature for 5 minutes and than at 39°C for another 25 minutes. Buffers were than replaced to be suitable for the streptavidine alkaline phosphatase conjugate incubations. After 30 minutes incubation at room temperature, conjugate was rinsed away and replaced by the substrate components for alkaline phosphatase, Nitro-Blue-Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate. After 30 minutes incubation at room temperature, probes where hybridization occurred became visible because of the purple brown precipitate at these positions.

After careful analysis of the 471 probes, the most specific and sensitive probes (n=46) were finally selected, covering the natural and drug-selected variability in the vicinity of aa. 30, 46, 48, 50, 54, 82, 84, and 90. Figure 2 shows the reactivity of the finally selected probes with the reference panel.

Example 3:

LiPA testing on clinical samples.

A total of 856 samples were tested on this selection of 46 specific probes. The geographical origin of these samples is as follows: USA:359 ; France: 154; UK:36; Brazil 58; Spain 35; Belgium 199; Luxembourg: 15.

From this population, a total of 144 samples were sequenced which allowed to separate the genotype B samples (94) from the non-B isolates (50). After analysis of these genotyped samples on LiPA, the genotypic reactivity on the selected probes was scored. Figures 4A to 4F show these results for the different codon positions and for the genotype B versus non-B group. From these tables, it is clear that there is little difference in sequence usage for the different codon positions with respect to specific reactivities at the different probes.

The total collection of 856 samples were then tested on the available 46 probes. After dissection of these reactivities over the different probes and different geographical origin, the picture looks as is presented in Figures 5A to 5F. Again here, the majority of the sequences used at the different codon positions is restricted to some very abundant wild type motifs. It is important to mention here that the majority of these samples are taken from patients never treated with protease inhibitors, en therefore, the majority of the reactivities is found in wild type motifs. Nevertheless, it is clear from some codon positions that the variability at some codon positions in the mutant motif might be considerable, and again, a continuos update on heavily treated patients is mandatory. Another issue is the amount of double blanc reactivities, which is in this approach reaching up to 5% in global; with some peak values for some countries for some codon positions: for example 13.8% for codon 82/85 in Brazil; and 18.1 % for codon 90 in Belgium.

REFERENCES

Asseline U, Delarue M, Lancelot G, Toulme F, Thuong N (1984) Nucleic acid-binding molecules with high affinity and base sequence specificity : intercalating agents covalently linked to oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA 81(11):3297-301.

Barany F. Genetic disease detection and DNA amplification using cloned thermostable ligase.

Proc Natl Acad Sci USA 1991; 88: 189-193.

Bej A, Mahbubani M, Miller R, Di Cesare J, Haff L, Atlas R. Mutiplex PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water. Mol Cell Probes 1990; 4:353-365.

Compton J. Nucleic acid sequence-based amplification. Nature 1991; 350: 91-92.

Condra *et al.* 1995. *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature 374: 569-571.

Condra *et al.* 1995. *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:** 569-571.

Duck P. Probe amplifier system based on chimeric cycling oligonucleotides. Biotechniques 1990; 9: 142-147.

Eberle *et al.* 1995. Resistance of HIV type 1 to proteinase inhibitor Ro 31-8959. AIDS Research and Human Retroviruses 11: 671-676.

Emini *et al.* 1994. Phenotypic and genotypic characterization of HIV-1 variants selected during treatment with the protease inhibitor L-735, L-524. Third International Workshop on HIV Drug Resistance, Kauai, Hawaii, USA.

Guatelli J, Whitfield K, Kwoh D, Barringer K, Richman D, Gengeras T. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci USA 1990; 87: 1874-1878.

Huff *et al.* 1991. HIV protease: a novel chemotherapeutic target for AIDS. J. Med. Chem. **34:** 2305-2314.

Hunter *et al.* 1994. Macromolecular interactions in the assembly of HIV and other retroviruses. Seminars in Virology **5:** 71-83.

Kempf *et al.* 1994. Pharmacokinetic and *in vitro* selection studies with ABT-538, a potent inhibitor of HIV protease with high oral bioavailability. 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, Fl, USA.

Kohl *et al.* 1988. Active human immunodeficienty virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA. **85:** 4686-4690.

Kwok S, Kellogg D, McKinney N, Spasic D, Goda L, Levenson C, Sinisky J. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency views type 1 model studies. Nucl. Acids Res. 1990; 18: 999.

Landgren U, Kaiser R, Sanders J, Hood L. A ligase-mediated gene detection technique. Science 1988; 241:1077-1080.

Lomeli H, Tyagi S, Printchard C, Lisardi P, Kramer F. Quantitative assays based on the use of replicatable hybridization probes. Clin Chem 1989; 35: 1826-1831.

Matsukura M, Shinozuka K, Zon G, Mitsuya H, Reitz M, Cohen J, Broder S (1987) Phosphorothioate analogs of oligodeoxynucleotides : inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 84(21):7706-10.

Meek *et al.* 1989. Proc. Natl. Acad. Sci. USA. **86:** 1841-1845.

Miller P, Yano J, Yano E, Carroll C, Jayaram K, Ts'o P (1979) Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside methylphosphonates. Biochemistry 18(23):5134-43.

Myers *et al.* 1996. Human retroviruses and AIDS 1996. Los Alamos Laboratory, Los Alamos,

N.M.

Navia *et al.* 1989. Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. Nature **337:** 615-620.

Nielsen P, Egholm M, Berg R, Buchardt O (1993) Sequence specific inhibition of DNA restriction enzyme cleavage by PNA. Nucleic-Acids-Res. 21(2):197-200.

Nielsen P, Egholm M, Berg R, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254(5037):1497-500.

Patick *et al.* 1996. Antiviral and risistance studies of AG1343, an orally bioavailable inhibitor of human immunodeficiency virus protease. Antimicrobial Agents and Chemotherapy 40: 292-297; 40: 1575 (erratum).

Peng *et al.* 1989. Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J. Virol. **63:** 2550-2556.

Rao *et al.* 1996. Structural and modeling analysis of the basis of viral resistance to VX-478. Fifth International Workshop on HIV Drug Resistance, Whistler, Canada, abstract n°: 22.

Saiki R, Walsh P, Levenson C, Erlich H. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes Proc Natl Acad Sci USA 1989; 86:6230-6234.

Schmit *et al.* 1996. Resistance-related mutations in the HIV-1 protease gene of patients treated for 1 year with the protease inhibitor ritonavir (ABT-538). AIDS **10:** 995-999.

Shinazi *et al.* 1997. Mutations in retroviral genes associated with drug resistance. International Antiviral News **5:** 129-142.

Stuyver L, Rossau R, Wyseur A, et al. Typing of hepatitis C virus isolates and characterization

of new subtypes using a line probe assay. J. Gen. Virol. 1993; 74 : 1093-1102.

Tisdale *et al.* 1994. Comprehensive analysis of HIV-1 variants individually selected for resistance to six HIV protease inhibitors. Third International Workshop on HIV Drug Resistance, Kauai, Hawaii, USA.

Wlodawer *et al.* 1993. Structure-based inhibitors of HIV-1 protease. Annu. Rev. Biochem. **62:**543-585.

Wlodawer *et al.* 1989. Science **245:** 616-621.

Wu D, Wallace B. The ligation amplification reaction (LAR) - amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 1989; 4:560-56

Table 1

	26 27 28 29 30 31 32 33 34 35	Tm	lengte
	ACA GGA GCA GAT GAT ACA GTA TTA GAA GAA		
pc30w25	GCA GAT GAT ACA GT	40	14
pc30w29	A GCG GAT GAT ACA	36	13
pc30w32	GCA GAT GAC ACA GT	42	14
pc30w36	GCA GAC GAT ACA GG	40	13
pc30m23	A GCA GAT AAT ACA GT	40	15

	44 45 46 47 48 49 50 51 52		
	CCA AAA ATG ATA GGG GGA ATT GGA GGT		
pc48w47	AAA ATG ATA GGG GGA	42	15
pc48w45	AAA ATG ATA GGA GGA ATT	42	16
pc48w72	A AAA ATA ATA GGG GGA	42	13
pc48m41	ATG ATA GTG GGA ATT	40	15

	48 49 50 51 52 53 54		
	GGG GGA ATT GGA GGT TTT ATC		
pc50w31	GGA ATT GGA GGT TTT	42	15
pc50w44	GGA ATT GGG GGT TTG	42	13
pc50w52	GA ATT GGA GGC TTG		13
pc50m37	GGG GGA GTT GGA	40	12

	51 52 53 54 55 56 57 58		
	GGA GGT TTT ATC AAA GTA AGA CAG		
pc54w3	GT TTT ATC AAA GTA AGA	42	17
pc54w34	GA GGT TTT ATC AAA GT	42	16
pc54w14	GGT TTT ATC AAG GTA A	42	16
pc54w19	A GGC TTT ATC AAA GTA	42	16
pc54w22	GA GGT TTT ATT AAA GTA	42	17
pc54w26	A GGT TTC ATT AAG GTA	42	16
pc54w27	GGT TTT ATT AAG GTA A	40	16
pc54m55	A GGT TTT GCC AAA GT	38	14
pc54m35	GGT TTT GTC AAA GTA	40	15
pc54m37	GGT TTT GTC AGA GTA	42	15

	78 79 80 81 82 83 84 85 86 87		
	GGA CCT ACA CCT GTC AAC ATA ATT GGA AGA		
pc82w91	ACA CCT GTC AAC ATA A	44	16
pc82w60	CA CCT GTC AAT ATA ATG	42	16
pc82w111	A CCG GTC AAC ATA ATT	44	16
pc82w89	ACA CCT GTT AAC ATA AG	42	16
pc82w42	CA CCT GTC AAC GTA	42	14
pc82m36	ACA CCT ACC AAC ATA	42	15
pc82m67	ACA CCT ACC AAC GT	42	14
pc82m38	ACA CCT TTC AAC ATA	40	15
pc82m105	ACG CCC TTC AAC ATA	44	15
pc82m127	CA CCT TTC AAC GTA ATG	44	16
pc82m40	ACA CCT GCC AAC ATA	44	15
pc82m63	CA CCT GCC AAT ATA AG	42	15
pc82m101	ACA CCT ATC AAC ATA ATG	44	17

	86 87 88 89 90 91 92 93 94		
	GGA AGA AAT CTG TTG ACT CAG ATT GGT		
pc90w27	AAT CTG TTG ACT CA	38	14

Table 4 cont

0w37	AAT CTG TTG ACT CAG ATG	42	15
0w39	GA ACT CTG TTG ACT C	44	15
0w50	AAT ATG TTG ACT CAG	40	15
0w52	AAT TTG TTG ACT CAG	40	15
0w69	GA AAC CTG TTG ACT	40	13
0w73	TG TTG ACA CAG CTT G	44	15
0w79	TG TTG ACC CAG ATT G	44	15
0m43	A AAT CTG ATG ACT CA	40	15
0m56	AAT ATG ATG ACC CAG	42	15

Table 2
Protease inhibitors

Compound	Amino acid change	Codon change
PROTEASE INHIBITORS		
A-77003	R8Q	CGA to CAA
	R8K	CGA to AAA
	V32I	GTA to ATA
	M46I	ATG to ATA
	M46L	ATG to TTC
	M46F	ATG to TTC
	M46V	ATG to GTG
	G48V	GGG to GTG
	A71V	GCT to GTT
	V82I	GTC to ATC
	V82A	GTC to GCC
	L63P	CTC to CCC
	A71T	GCT to ACT
	A71V	GCT to GTT
	G73S	GGT to GCT
	V82A	GTC to GCC
	V82F	GTC to TTC
	V82T	GTC to ACC
	I84V	ATA to GTA
	L90M	TTG to ATG
P9941	V82A	GTC to GCC
Ro 31-8959 (saquinavir)	L10I	CTC to ATC
	G48V	GGG to GTG
	I54V	ATC to GTC
	I54V	ATA to GTA
	G73S	GGT to AGT
	V82A	GTC to GCC
	I84V	ATA to GTA
	L90M	TTG to ATG
RPI-312	I84V	ATA to GTA
SC-52151	L24V	TTA to GTA
	G48V	GGG to GTG
	A71V	GCT to GTT
	V75I	GTA to ATA
	P81T	CCT to ACT
	V82A	GTC to GCC
	N88D	AAT to GAT
SC-55389A	L10F	CTC to CGC
	N88S	AAT to AGT
SKF108842	V82T	GTC to ACC
	I84V	ATA to GTA
SKF108922	V82A	GTC to GCC
	V82T	GTC to ACC
VB 11,328	L10F	CTC to GGC
	M46I	ATG to ATA
	I47V	ATA to CTA
	I50V	ATT to GTT
	I84V	ATA to GTA
VX-478 (141W94)	L10F	CTC to CGC
	M46I	ATG to ATA
	I47V	ATA to CTA
	I50V	ATT to GTT
	I84V	ATA to GTA
XM323	L10F	CTC to CGC
	K45I	AAA to ATA
	M46L	ATG to CTG
	V82A	GTC to GCC
	V82I	GTC to ATC
	V82F	GTC to TTC
	I84V	ATA to GTA
	L97V	TTA to GTA
	I82T	ATC to ACC
A-75925	V32I	GTA to ATA
ABT-538 (ritonavir)	K20R	AAG to AAA
	L33F	TTA to TTC
	M36I	ATG to ATA
	M46I	ATG to ATA
	I54L	ATC to ?
	I54V	ATC to GTC
	A71V	GCT to GTT
	V82F	GTC to TTC
	V82A	GTC to GCC
	V82T	GTC to ACC
	V82S	GTC to TCC
	I84V	ATA to GTA
	L90M	TTG to ATG
AG1343 (nelfinavir)	D30N	GAT to AAT
	M36I	
	M46I	ATG to ATA
	L63P	CTC to CCC
	A71V	GCT to GTT
	V77I	
	I84V	ATA to GTA
	N88D	
	L90M	TTG to ATG
BILA 1906 BS	V32I	GTA to ATA
	M46I	ATG to ATA
	M46L	ATG to TTG
	A71V	GCT to GTT
	I84A	ATA to GCA
	I84V	ATA to GTA
BILA 2011 (palinavir)	V32I	GTA to ATA
	A71V	GCT to GTT
	I84A	ATG to ATA
	L63P	CTC to CCC
BILA 2185 BS	L23I	CTA to ATA
BMS 186,318	A71T	GCT to ACT
	V82A	GTC to GCC
DMP 450	L10F	CTC to TTC
	M46I	ATG to ATA
	D60E	GAT to GAA
	I84V	ATA to GTA
KNI-272	V32I	GTA to ATA
MK-639 (L-735,524, indinavir)	L10I	CTC to ATC
	L10R	CTC to CGC
	L10V	CTC to GTC
	K20M	AAG to ATG
	K20R	AAG to AAA
	L24I	TTA to ATA
	V32I	GTA to ATA
	M46I	ATG to ATA
	M46L	ATG to TTG
	I54V	ATC to GTC

Table 3

	26	27	28	29	30	31	32	33	34	35	length	Seq ID
	ACA	GGA	GCA	GAT	GAT	ACA	GTA	TTA	GAA	GAA		
P30w1		A	GCA	GAT	GAT	ACA	GTA	TT			18	7
P30w2		GA	GCA	GAT	GAT	ACA	GTA	TT			19	8
P30w3		A	GCA	GAT	GAT	ACA	GTA	TTA			19	9
P30w4		GGA	GCA	GAT	GAT	ACA	GTA	TT			20	10
P30w5		GGA	GCA	GAT	GAT	ACA	GTA	TTA			21	11
P30w6	ACA	GGA	GCA	GAT	GAT	ACA					18	12
P30w7	CA	GGA	GCA	GAT	GAT	ACA	GT				19	13
P30w8	A	GGA	GCA	GAT	GAT	ACA	GTA	TG			20	14
P30w9		GGA	GCA	GAT	GAT	ACA	GTA	TG			19	15
P30w10	ACA	GGA	GCA	GAT	GAT	ACA	GG				19	16
P30m11		A	GCA	GAT	AAT	ACA	GTA	TT			18	17
P30m12		GA	GCA	GAT	AAT	ACA	GTA	TT			19	18
P30m13		A	GCA	GAT	AAT	ACA	GTA	TTA			19	19
P30m14		GGA	GCA	GAT	AAT	ACA	GTA	TT			20	20
P30m15		GGA	GCA	GAT	AAT	ACA	GTA	TTA			21	21
P30m15	ACA	GGA	GCA	GAT	AAT	ACA					18	22
P30m17	CA	GGA	GCA	GAT	AAT	ACA	GT				19	23
P30m18	A	GGA	GCA	GAT	AAT	ACA	GTA	TG			20	24
P30m19		GGA	GCA	GAT	AAT	ACA	GTA	TG			19	25
P30m20	ACA	GGA	GCA	GAT	AAT	ACA	GG				19	26
P30w21		A	GCA	GAT	GAT	ACA	GT				15	27
P30w22		A	GCA	GAT	GAT	ACA	GTA	G			16	28
P30m23		A	GCA	GAT	AAT	ACA	GTA				15	29
P30m24		A	GCA	GAT	AAT	ACA	GTA	G			16	30
P30w25			GCA	GAT	GAT	ACA	GT				14	31
P30w26		A	GCA	GAT	GAT	ACA	GG				14	32
P30w27			CA	GAT	GAT	ACA	GT				13	33
P30w28		GA	GCG	GAT	GAT	ACA					14	34
P30w29		A	GCG	GAT	GAT	ACA					13	35
P30m30			GCA	GAT	AAT	ACA	GTA				15	36
P30m31			GCA	GAT	AAT	ACA	GT				14	37
P30w32			GCA	GAT	GAC	ACA	GT				14	38
P30w33			CA	GAT	GAC	ACA	GTA	G			14	39
P30w34			CA	GAT	GAT	ACA	ATA	TT			16	40
P30w35			GCA	GAT	GAT	ACA	ATA	TG			16	41
P30w36			GCA	GAC	GAT	ACA	GG				13	42
P30w37			GCA	GAC	GAT	ACA	GT				14	43
P30w38			A	GAT	GAT	ACA	ATA	TT			15	44
P30w39			A	GAT	GAT	ACA	ATA	TTA			16	45
P30w40			GCA	GAT	GAT	ACA	ATA				15	46

54

Table 3 cont

	44	45	46	47	48	49	50	51	52	53	54	length	Seq ID
	CCA	AAA	ATG	ATA	GGG	GGA	ATT	GGA	GGT	TTT	ATC		
P48w1				GTA	GGG	GGA	ATT	GGA	GGT	GG		18	47
P48w2				GTA	GGG	GGA	ATT	GGA	GGT	TG		19	48
P48w3				GTA	GGG	GGA	ATT	GGA	GGT	TTG		20	49
P48w4				GTA	GGG	GGA	ATT	GGA	GGT	TTT		21	50
P48w5			G	GTA	GGG	GGA	ATT	GGA	GGT	TTG		21	51
P48w6			ATG	GTA	GGG	GGA	ATT	GGA				18	52
P48w7			ATG	GTA	GGG	GGA	ATT	GGA	G			19	53
P48w8		A	ATG	GTA	GGG	GGA	ATT	GGA				19	54
P48w9		A	ATG	GTA	GGG	GGA	ATT	GGA	G			20	55
P48w10		A	ATG	GTA	GGG	GGA	ATT	GGA	GGG	GG		22	56
P48w21			ATA	ATA	GGG	GGA	ATT	GGA				18	57
P48w22			ATG	ATA	GGG	GGA	ATT	GGA				18	58
P48w23		A	ATA	ATA	GGG	GGA	ATT	GGA				19	59
P48w24		A	ATG	ATA	GGG	GGA	ATT	GGA				19	60
P48w25				ATA	GGG	GGA	ATT	GGA	GGT	GG		18	61
P48w26				ATA	GGG	GGA	ATT	GGA	GGT	TG		19	62
P48w28				ATA	GGG	GGA	ATT	GGA	GGT	TTG		20	63
P48w29				ATA	GGG	GGA	ATT	GGA	GGT	TTT		21	64
P48m11				GTA	GTG	GGA	ATT	GGA	GGT	GG		18	65
P48m12				GTA	GTG	GGA	ATT	GGA	GGT	TG		19	66
P48m13				GTA	GTG	GGA	ATT	GGA	GGT	TTG		20	67
P48m14				GTA	GTG	GGA	ATT	GGA	GGT	TTT		21	68
P48m15			G	GTA	GTG	GGA	ATT	GGA	GGT	TTG		21	69
P48m16			ATG	GTA	GTG	GGA	ATT	GGA				18	70
P48m17			ATG	GTA	GTG	GGA	ATT	GGA	G			19	71
P48m18		A	ATG	GTA	GTG	GGA	ATT	GGA				19	72
P48m19		A	ATG	GTA	GTG	GGA	ATT	GGA	G			20	73
P48m20		A	ATG	GTA	GTG	GGA	ATT	GGA	GGG	GG		22	74
P48m29				ATA	GTG	GGA	ATT	GGA	GGT	GG		18	75
P48m30				ATA	GTG	GGA	ATT	GGA	GGT	TG		19	76
P48m31			ATG	ATA	GTG	GGA	ATT	GGA				18	77
P48m32			ATG	ATA	GTG	GGA	ATT	GGA	G			19	78
P48m33		A	ATG	ATA	GTG	GGA	ATT	GGA				19	79
p48w34			G	ATA	GGG	GGA	ATT	G				14	80
p48w35			TG	ATA	GGG	GGA	ATT	G				15	81
p48w36			TG	ATA	GGG	GGA	ATT	GG				16	82
p48w37			ATG	ATA	GGG	GGA	ATT					15	83
p48m38			G	ATA	GTG	GGA	ATT	G				14	84
p48m39			TG	ATA	GTG	GGA	ATT	G				15	85
p48m40			TG	ATA	GTG	GGA	ATT	GG				16	86
p48m41			ATG	ATA	GTG	GGA	ATT					15	87
p48w42			ATA	ATA	GGG	GGA	ATT					15	88
p48w43			TG	ATA	GGG	GGA	GTT					14	89
p48w44			G	ATA	GGG	GGA	GTT	G				14	90
p48w45		A	ATG	ATA	GGA	GGA	ATT					16	91
p48w46			ATG	ATA	GGG	GGA	ATT					15	92
p48w47		AAA	ATG	ATA	GGG	GGA						15	93
p48w48	A	AAA	ATG	ATA	GGG	GG						15	94
p48w49		AA	ATG	ATA	GGG	GGA	AG					15	95
p48w50		AAA	ATA	ATA	GGG	GGA	AG					16	96
p48w51		AAA	ATA	AAA	AT							15	97
p48m52		AAA	ATG	ATA	GTG	GGA	AG					16	98
p48w52b		AAA	TTG	ATA	GGG	GG						14	99
p48m53		AAA	ATG	ATA	GTG	GGA						15	100

8w53b	AAA TTG ATA GGG GGA	15	101
48w54	CA AAA TTG ATA G	15	102
48w55	ATG GTA GGG GGA ATT	15	103
48w56	AA ATG GTA GGG GGA	14	104
48w57	A AAA ATG GTA GGG G	14	105
48w58	ATG ATA GGG GAA ATT	15	106
48w59	ATA GGG GAA ATT GGA	15	107
48w60	ATA GGG GAA ATT GGA G	16	108
48w61	ATG ATA GGG GGG ATT	15	109
48w62	ATA GGG GGG ATT GG	14	110
48w63	A GGG GGG ATT GGA	13	111
48m64	AAA ATA ATA GTG GGA	15	112
48m65	A AAA ATA ATA GTG GGA	16	113
48m66	CA AAA ATA ATA GTG GG	16	114
48m67	AAA TTG ATA GTG GGA	15	115
48m68	A AAA TTG ATA GTG GGA	16	116
48m69	CA AAA TTG ATA GTG G	15	117
48w70	AAA ATG ATA GGG GG	14	118
48w71	A AAA ATG ATA GGG G	14	119
8w72	A AAA ATA ATA GGG GGA	16	120

Tableau

	45 46 47 48 49 50 51 52 53 54	length	Seq ID
	AAA ATG GTA GGG GGA ATT GGA GGT TTT ATC		
P50w1	GGG GGA ATT GGA GGT TTT	18	121
P50w2	A GGG GGA ATT GGA GGT TTT	19	122
P50w3	TA GGG GGA ATT GGA GGT TTT	20	123
P50w4	A GGG GGA ATT GGA GGT TTT AG	20	124
P50w5	TA GGG GGA ATT GGA GGT TTT AG	21	125
P50w6	GTA GGG GGA ATT GGA GGT TGG	19	126
P50w7	G GTA GGG GGA ATT GGA GGT TGG	20	127
P50w8	GTA GGG GGA ATT GGA GGT TTG	20	128
P50w9	GTA GGG GGA ATT GGA GGT TTT	20	129
P50w10	TG GTA GGG GGA ATT GGA GGT GG	20	130
p50w21	GG GGA ATT GGA GGT TTT	17	131
P50w22	GG GGA ATT GGA GGT TTG	16	132
P50w23	GG GGA ATT GGA GGT TTT AG	18	133
P50w24	GG GGA ATT GGA GGT TG	15	134
P50w25	G GGA ATT GGA GGT TTT AT	18	135
P50w26	GG GGA ATT GGA GGT TTT	17	136
P50m11	GGG GGA GTT GGA GGT TTT	18	137
P50m12	A GGG GGA GTT GGA GGT TTT	19	138
P50m13	TA GGG GGA GTT GGA GGT TTT	20	139
P50m14	A GGG GGA GTT GGA GGT TTT AG	20	140
P50m15	TA GGG GGA GTT GGA GGT TTT AG	21	141
P50m16	GTA GGG GGA GTT GGA GGT TGG	19	142
P50m17	G GTA GGG GGA GTT GGA GGT TGG	20	143
P50m18	GTA GGG GGA GTT GGA GGT TTG	20	144
P50m19	GTA GGG GGA GTT GGA GGT TTT ATC	21	145
P50m20	TG GTA GGG GGA GTT GGA GGT GG	20	146
P50m27	GG GGA GTT GGA GGT TTG	19	147
P50m28	GG GGA GTT GGA GGT TTT AG	18	148
P50m29	GG GGA GTT GGA GGT TG	15	149
P50m30	G GGA GTT GGA GGT TTT AT	18	150
p50w31	GGA ATT GGA GGT TTT	15	151
p50w32	G GGA ATT GGA GGT TGG	15	152
p50m33	GGA GTT GGA GGT TTT	15	153
p50m34	G GGA GTT GGA GGT TGG	14	154
p50m35	GGG GGA GTT GGA G	13	155
p50m36	GG GGA GTT GGA G	12	156
p50m37	GGG GGA GTT GGA	12	157
p50w38	GGA ATT GGG GGT TTG	14	158
p50w39	GA ATT GGG GGT TTT	14	159
p50w40	GA ATT GGG GGT TTT AG	15	160
p50w41	GGA ATT GGG GGT TG	13	161
p50w42	GGA ATT GGG GGT G	12	162
p50w43	GA ATT GGG GGT TG	12	163
p50w44	GA ATT GGG GGT TTG	13	164
p50w45	GGG GGA ATT GCA G	13	165
p50w46	GGA ATT GCA GGT TG	14	166
p50w47	GGA ATT GCA GGT G	13	167
p50w48	GGA ATT GGA GGG TTG	14	168
p50w49	GA ATT GGA GGG TTG	13	169
p50w50	GA ATT GGA GGG TTT	14	170
p50w51	GGA ATT GGA GGC TTG	14	171
p50w52	GA ATT GGA GGC TTG	13	172
p50w53	GA ATT GGA GGC TTT	14	173
p50m54	GGA GTT GGA GGT TTG	15	174

Table 3 cont

b50m55	GA GTT GGA GGT TTT	14	175

Table 3 cont

	51	52	53	54	55	56	57	58	length	Seq ID
	GGA	GGT	TTT	ATC	AAA	GTA	AGA	CAG		
p54w1		GGT	TTT	ATC	AAA	GTA	A		16	176
p54w2		GT	TTT	ATC	AAA	GTA	AG		16	177
p54w3		GT	TTT	ATC	AAA	GTA	AGA		17	178
p54w4		T	TTT	ATC	AAA	GTA	AGA		16	179
p54w5		GGT	TTT	ATC	AAA	GTA			15	180
p54w6		GT	TTT	ATC	AAA	GTA			15	181
p54m7		GGT	TTT	GCC	AAA	GTA			15	182
p54m8		GT	TTT	GCC	AAA	GTA	A		15	183
p54m9		GT	TTT	GCC	AAA	GTA	AG		16	184
p54m10		T	TTT	GCC	AAA	GTA	AGA		16	185
p54m11		GGT	TTT	GCC	AAA	GT			14	186
p54m12		GT	TTT	GCC	AAA	GTA			14	187
p54w13		GT	TTT	ATC	AAG	GTA	AA		16	188
p54w14		GGT	TTT	ATC	AAG	GTA	A		16	189
p54w15	A	GGT	TTT	ATC	AAG	GTA			16	190
p54w16		GT	TTT	ATC	AAA	GTC	AGA		17	191
p54w17			TTT	ATC	AAA	GTC	AGA	C	16	192
p54w18	A	GGC	TTT	ATC	AAA	GTA	A		17	193
p54w19	A	GGC	TTT	ATC	AAA	GTA			16	194
p54m20	A	GGT	TTT	ATT	AAA	GTA	A		17	195
p54m21		GGT	TTT	ATT	AAA	GTA	AG		17	196
p54w22	GA	GGT	TTT	ATT	AAA	GTA			17	197
p54m22	GA	GGT	TTT	ATT	AAA	GTA			17	198
p54m23		GGT	TTT	ATT	GGT	TTT	AT		16	199
p54m24		GGT	TTC	ATT	AAG	GTA			15	200
p54m25		GGT	TTC	ATT	AAG	GTA	A		16	201
p54w26	A	GGT	TTC	ATT	AAG	GTA			16	202
p54m26	A	GGT	TTC	ATT	AAG	GTA			16	203
p54w27		GGT	TTT	ATT	AAG	GTA	A		16	204
p54m27		GGT	TTT	ATT	AAG	GTA	A		16	205
p54m28	A	GGT	TTT	ATT	AAG	GTA			16	206
p54m29	GA	GGT	TTT	ATT	AAG	GT			16	207
p54m30		GGT	TTT	ATT	AAG	GTA	AG		17	208
p54w31		GGT	TTT	ATC	AAA	GTA	A		16	209
p54w32	A	GGT	TTT	ATC	AAA	GTA	A		17	210
p54w33	A	GGT	TTT	ATC	AAA	GTA			16	211
p54w34	GA	GGT	TTT	ATC	AAA	GT			16	212
p54m35		GGT	TTT	GTC	AAA	GTA			15	213
p54m36		GGT	TTT	GTC	AAA	GTA	A		16	214
p54m37		GGT	TTT	GTC	AGA	GTA			15	215
p54m38		GGT	TTT	GTC	AGA	GTA	A		16	216
p54w39		GGG	TTT	ATC	AAA	GTA			15	217
p54w40		GGG	TTT	ATC	AAA	GTA	A		16	218
p54w41		GGC	TTC	ATC	AAA	GT			14	219
p54w42	GA	GGC	TTC	ATC	AAA				14	220
p54m48		GGT	TTT	GTC	AAA	GT			14	221
p54m49		GT	TTT	GTC	AGA	GTA			14	222
p54m50		GGT	TTT	GTC	AGA	GT			14	223
p54w51	A	GGT	TTA	ATC	AAA	GTA			16	224
p54w52	GA	GGT	TTA	ATC	AAA	GT			16	225
p54m53		GGT	TTT	ACC	AAA	GTA			15	226
p54m54		GGT	TTT	ACC	AAA	GT			14	227

Table 3 cont

	78	79	80	81	82	83	84	85	86	87	length	Seq ID
	GGA	CCT	ACA	CCT	GTC	AAC	ATA	ATT	GGA	AGA		
P82w1			CCT	ACA	CCT	GTC	AAC	ATA	AG		19	228
P82w2			CCT	ACA	CCT	GTC	AAC	ATA	ATG		20	229
P82w3			CCT	ACA	CCT	GTC	AAC	ATA	ATT		21	230
P82w4		A	CCT	ACA	CCT	GTC	AAC	ATA	AG		20	231
P82w5		A	CCT	ACA	CCT	GTC	AAC	ATA	ATG		21	232
P82w6		A	CCT	ACA	CCT	GTC	AAC	ATA			19	233
P82w7		GA	CCT	ACA	CCT	GTC	AAC	ATA			20	234
P82w8				CA	CCT	GTC	AAC	ATA	ATT	GGA	20	235
P82w9				A	CCT	GTC	AAC	ATA	ATT	GGA A	20	236
P82w10				ACA	CCT	GTC	AAC	ATA	ATT	GG	20	237
P82W21				A	CCT	GTC	AAC	ATA	ATT	GGA	19	238
P82m11			CCT	ACA	CCT	ACC	AAC	ATA	AG		19	239
P82m12			CCT	ACA	CCT	ACC	AAC	ATA	ATG		20	240
P82m13			CCT	ACA	CCT	ACC	AAC	ATA	ATT		21	241
P82m14		A	CCT	ACA	CCT	ACC	AAC	ATA	AG		20	242
P82m15		A	CCT	ACA	CCT	ACC	AAC	ATA	ATG		21	243
P82m16		A	CCT	ACA	CCT	ACC	AAC	ATA			19	244
P82m17		GA	CCT	ACA	CCT	ACC	AAC	ATA			20	245
P82m18				CA	CCT	ACC	AAC	ATA	ATT	GGA	20	246
P82m19				A	CCT	ACC	AAC	ATA	ATT	GGA A	20	247
P82m20				ACA	CCT	ACC	AAC	ATA	ATT	G	19	248
P82m22			CCT	ACA	CCT	TTC	AAC	ATA	ATT		21	249
P82m23			CCT	ACA	CCT	GCC	AAC	ATA	ATT		21	250
P82m24			CCT	ACA	CCT	TCC	AAC	ATA	ATT		21	251
P82m25				A	CCT	TTC	AAC	ATA	ATT	GGA A	20	252
P82m26				A	CCT	GCC	AAC	ATA	ATT	GGA A	20	253
P82m27				A	CCT	TTC	AAC	ATA	ATT	GGA A	20	254
P82m28				A	CCT	ACC	AAC	ATA	ATT		16	255
P82m29				A	CCT	TTC	AAC	ATA	ATT	GGA	19	256
P82m30				A	CCT	GCC	AAC	ATA	ATT	GGA	19	257
P82m31				A	CCT	TCC	AAC	ATA	ATT	GGA	19	258
P82w32			T	ACA	CCT	GTC	AAC	AT			15	259
P82w33			T	ACA	CCT	GTC	AAC	ATA			16	260
P82w34				ACA	CCT	GTC	AAC	ATA			15	261
P82w35				CA	CCT	GTC	AAC	ATA			14	262
P82m36				ACA	CCT	ACC	AAC	ATA			15	263
P82m37				CA	CCT	ACC	AAC	ATA			14	264
P82m38				ACA	CCT	TTC	AAC	ATA			15	265
P82m39				CA	CCT	TTC	AAC	ATA			14	266
P82m40				ACA	CCT	GCC	AAC	ATA			15	267
P82m41				CA	CCT	GCC	AAC	ATA			14	268
P82w42				CA	CCT	GTC	AAC	GTA			14	269
P82w43				CA	CCT	GTC	AAC	GT			13	270
P82w44			CCT	ACA	CCT	GTC	AAC				15	271
P82w45			T	ACG	CCT	GTC	AAC	AT			15	272
P82w46			CT	ACG	CCT	GTC	AAC	AG			15	273
P82m47				ACA	CCT	TCC	AAC	ATA			15	274
P82m48				CA	CCT	TCC	AAC	ATA			14	275
P82m49				ACA	CCT	TCC	AAC	AT			14	276
P82m50				ACA	CCT	ATC	AAC	ATA			15	277
P82m51				CA	CCT	ATC	AAC	ATA	AG		15	278
P82m52				CA	CCT	ATC	AAC	ATA	ATG		16	279
P82m53				A	CCT	ATC	AAC	ATA	ATG		15	280
P82w54					CCT	GTC	AAC	ATA	ATT		15	281

Table 3 cont'd

P82w55	CCT GTT AAC ATA ATT G	16	282
P82w56	A CCT GTT AAC ATA ATG	15	283
P82w57	CCG GTC AAC ATA ATT	15	284
P82w58	ACG CCT GTC AAC AT	14	285
P82w59	CCT GTC AAT ATA ATT	15	286
P82w60	CA CCT GTC AAT ATA ATG	16	287
P82w61	ACA CCT GTC AAT ATA AG	16	288
P82m62	CCT GCC AAT ATA ATT	15	289
P82m63	CA CCT GCC AAT ATA AG	15	290
P82m64	CCT ACC AAC GTA ATT	15	291
P82m65	CCT ACC AAC GTA ATG	14	292
P82m66	CA CCT ACC AAC GTA	14	293
P82m67	ACA CCT ACC AAC GT	14	294
P82m68	CCT TTC AAC GTA ATT	15	295
P82m69	CA CCT TTC AAC GTA AG	15	296
P82m70	ACA CCT TTC AAC GTA	15	297
P82m71	A CCT TTC AAC GTA ATG	15	298
p82w72	CT GTC AAT ATA ATT G	15	299
p82w73	CCT GTC AAT ATA ATT G	16	300
p82w74	A CCT GTC AAT ATA ATT	16	301
p82w75	C[illegible] GTC AAT ATA ATT GG	16	302
p82w76	CCT ACG CCT GTC AA	14	303
p82w77	CT ACG CCT GTC AAC	14	304
p82w78	A CCT ACG CCT GTC AA	15	305
p82w79	A CCT ACG CCT GTC A	14	306
p82w80	T ACA CCG GTC AAC A	14	307
p82w81	CT ACA CCG GTC AA	13	308
p82w82	CCT ACA CCG GTC A	13	309
p82w83	CA CCT GTC AAC ATA A	15	310
p82w84	A CCT GTC AAC ATA AT	15	311
p82w85	CT ACA CCT GTC AAC A	15	312
p82w86	ACA CCT GTC AAC AT	14	313
p82w87	A CCT GTT AAC ATA ATT G	17	314
p82w88	CA CCT GTT AAC ATA AG	15	315
p82w89	ACA CCT GTT AAC ATA AG	16	316
p82w90	TCA CCT GTC AAC ATA	14	317
p82w91	ACA CCT GTC AAC ATA A	16	318
p82w92	CA CCT GTC AAC ATA AT	16	319
p82w93	CCT GTC AAC ATA ATT	15	320
p82w94	A CCT GTC AAC ATA ATT	16	321
p82w95	CCT GTC AAC ATA ATT G	16	322
P82w96	CCT ACA CCT GTC AA	14	323
p82w97	T GTC AAC ATA ATT GG	15	324
p82w98	T GTC AAC ATA ATT GGA	16	325
p82m99	ACA CCT TTC AAC ATA A	16	326
p82m100	T ACA CCT TTC AAC ATA	16	327
p82m101	ACA CCT ATC AAC ATA ATG	17	328
P82m102	ACA CCT ATC AAC ATA AG	16	329
p82m103	CA CCT GCC AAT ATA ATG	16	330
p82m104	ACA CCT GCC AAT ATA AG	16	331
p82m105	ACG CCC TTC AAC ATA	15	332
p82m106	CG CCC TTC AAC ATA AG	15	333
p82m107	T ACG CCC TTC AAC AT	15	334
p82w108	CT ACA CCG GTC AAC	14	335
p82w109	CCT ACA CCG GTC AA	14	336
p82w110	A CCG GTC AAC ATA ATG	15	337

32w111	A CCG GTC AAC ATA ATT	16	338
32w112	CT ACA CCA GTC AAC	14	339
32w113	CT ACA CCA GTC AAC A	15	340
32w114	ACA CCA GTC AAC ATA	15	341
32w115	ACA CCA GTC AAC ATA AG	16	342
32w116	T ACG CCT GTC AAC AT	15	343
32w117	ACG CCT GTC AAC ATA	15	344
32w118	T ACG CCT GTC AAC A	14	345
32m119	CCT ACA CCT TTC AAC	15	346
32m120	CT ACA CCT TTC AAC	14	347
32m121	A CCT ACA CCT TTC AA	15	348
32w122	ACG CCT GTC AAC ATA AGG	16	349
32w123	T ACG CCT GTC AAC ATA	16	350
32w124	CG CCT GTC AAC ATA AGG	15	351
32m125	T ACA CCT TTC AAC GTA	16	352
32m126	ACA CCT TTC AAC GTA AGG	16	353
32m127	CA CCT TTC AAC GTA ATG	16	354
32m128	A CCT TTC AAC GTA ATT	16	355
32o129	C AAC GTA ATT GGA AGA	16	356
32o130	C AAC GTA ATT GGA AG	15	357

Table 3 cont

	86	87	88	89	90	91	92	93	94	length	Seq ID
	GGA	AGA	AAT	CTG	TTG	ACT	CAG	ATT	GGT		
P90w1		A	AAT	CTG	TTG	ACT	CAG			16	358
P90w2		GA	AAT	CTG	TTG	ACT	CAG			17	359
P90w3		GA	AAT	CTG	TTG	ACT	CAG	AGG		18	360
P90w4		A	AAT	CTG	TTG	ACT	CAG	AGG		17	361
P90w5		AGA	AAT	CTG	TTG	ACT	CAG	AGG		19	362
P90w6		AGA	AAT	CTG	TTG	ACT	CAG	ATG		20	363
P90w7		AGA	AAT	CTG	TTG	ACT	CAG	ATT		21	364
P90w8		AGA	AAT	CTG	TTG	ACT	CAG	ATTGG		20	365
P90w9	GA	AGA	AAT	CTG	TTG	ACT	CAG	AGG		21	366
P90w10	A	AGA	AAT	CTG	TTG	ACT	CAG	ATG		21	367
P90m11		AGA	AAT	CTG	ATG	ACT	CAG	ATG		20	368
P90m12		AGA	AAT	CTG	ATG	ACT	CAG	ATT		21	369
P90m13	A	AGA	AAT	CTG	ATG	ACT	CAG	AGG		20	370
P90m14	GA	AGA	AAT	CTG	ATG	ACT	CAG	AGG		21	371
P90m15	A	AGA	AAT	CTG	ATG	ACT	CAG	ATG		21	372
P90m16	GA	AGA	AAT	CTG	ATG	ACT	CAG	ATT		20	373
P90m17	GGA	AGA	AAT	CTG	ATG	ACT	CAG			21	374
P90m18	A	AGA	AAT	CTG	ATG	ACT	CAG			19	375
P90m19		A	AAT	CTG	ATG	ACT	CAG	ATT	GG	21	376
P90m20		A	AAT	CTG	ATG	ACT	CAG	ATT	G	20	377
P90m21		A	AAT	CTG	ATG	ACT	CAG	CTT	G	20	378
P90m22		A	AAT	CTG	ATG	ACT	CAG	CTT		19	379
P90m23			AAT	CTG	ATG	ACT	CAG	CTT	G	18	380
P90w24		A	AAT	CTG	TTG	ACT	CAG	CTT	G	20	381
P90w25		A	AAT	CTG	TTG	ACT	CAG	CTT		19	382
P90w26			AAT	CTG	TTG	ACT	CAG	CTT	G	19	383
P90w27			AAT	CTG	TTG	ACT	CA			14	384
P90w28			AAT	CTG	TTG	ACT	CAG			15	385
P90w29		A	AAT	CTG	TTG	ACT	CA			15	386
P90w30		A	AAT	CTG	TTG	ACT	CAG			16	387
P90m31			AAT	CTG	ATG	ACT	CA			14	388
P90m32			AAT	CTG	ATG	ACT	CAG			15	389
P90m33		A	AAT	CTG	ATG	ACT	CA			15	390
P90m34		A	AAT	CTG	ATG	ACT	CAG			16	391
P90w35		GA	AAT	CTG	TTG	ACT	C			15	392
P90w36		GA	ACT	CTG	TTG	ACT	C			15	393
P90w37			T	CTG	TTG	ACT	CAG	ATG		15	394
P90w38		GA	AAT	CTG	TTG	ACT	C			15	395
P90w39		GA	ACT	CTG	TTG	ACT	C			15	396
P90w40		A	AAT	CTG	TTG	ACT	CA			15	397
P90w41			AAT	CTG	TTG	ACT	CAG			15	398
P90m42			AAT	CTG	ATG	ACT	CAG			15	399
P90m43		A	AAT	CTG	ATG	ACT	CA			15	400
P90w44			AT	CTG	TTG	ACT	CAG	AG		15	401
P90w45				CTG	TTG	ACT	CAG	ATT		15	402
P90w46		AGA	AAT	CTG	TTG	ACT				15	403
P90m47			AT	CTG	ATG	ACT	CAG	AG		15	404
P90m48				CTG	ATG	ACT	CAG	ATT		15	405
P90m49		AGA	AAT	CTG	ATG	ACT	CA			17	406
P90w50			AAT	ATG	TTG	ACT	CAG			15	407
P90w51		GA	AAT	ATG	TTG	ACT	CA			16	408
P90w52			AAT	TTG	TTG	ACT	CAG			15	409
P90w53		GA	AAT	TTG	TTG	ACT	CA			16	410
P90w54			AAT	ATG	TTG	ACC	CAG			15	411

Table 3 cont

90w55	A AAT ATG TTG ACC CA	15	412
90m56	AAT ATG ATG ACC CAG	15	413
90m57	A CAG ATG ATG ACC CA	15	414
90w58	AAC ATG TTG ACT CAG	15	415
90w59	A AAC ATG TTG ACT CAG	15	416
90w60	TG TTG ACT CAG CTT	14	417
90w61	CTG TTG ACT CAG CTG	14	418
90m62	CT ATG ACT CAG CTT	14	419
90m63	CTG ATG ACT CAG C-G	14	420
90w64	TG ACT ACA CAG CTT	14	421
90w65	CTG TTG ACA CAG C-G	14	422
90w66	AAT CTG TTG ACA CAG	15	423
90w67	AAC CTG TTG ACT CA	13	424
90w68	A AAC CTG TTG ACT C	13	425
90w69	GA AAC CTG TTG ACT	13	426
90w70	TG TTG ACT CAG ATT G	15	427
90w71	TG TTG ACT CAG ATT GGG	16	428
90w72	G TTG ACT CAG ATT GGG	15	429
90w73	TG TTG ACA CAG CTT G	15	430
[redacted]w74	CTG TTG ACA CAG CTT	15	431
[redacted]w75	G TTG ACA CAG CTT GGG	15	432
90w76	TG TTG ACT CAG CTT G	15	433
90w77	G TTG ACT CAG ATG	15	434
90w78	G TTG ACT CAG CTT G	14	435
90w79	TG TTG ACC CAG ATT G	15	436
90w80	G TTG ACC CAG ATT G	14	437
90w81	G TTG ACC CAG ATT GGG	15	438
90m82	TG ATG ACT CAG ATT G	15	439
90m83	TG ATG ACT CAG ATT GGG	16	440
90m84	G ATG ACT CAG ATT GGG	15	441
90m85	G ATG ACT CAG ATT GGT	16	442
90m86	CTG ATG ACT CAG CTT	15	443
90m87	TG ATG ACT CAG CTT G	15	444
90w88	A AAT CTG TTG ACT CA	15	445
90w89	A AAT CTG TTG ACT CA	15	446
90w90	A AAT CTG TTG ACT CA	15	447
90w100	AAT CTG ATG ACT CAG	15	448
90m92	A AAT CTG ATG ACT CA	16	449
[redacted]m93	GA AAT CTG ATG ACT C	15	450
[redacted]m94	CTG ATG ACT CAG ATG	15	451
90m95	AGA AAT ATG ATG	15	452
90m96	A AGA AAT ATG ATG ACT	16	453
90m97	A AGA AAT CTG ATG ACT	16	454
90m98	A AGA AAT ATA ATG ACT	16	455
90m99	A AAT ATA ATG ACT CAG	16	456
90ml00	AAT ATG ATG ACC CAG	15	457
90ml01	AAC CTG ATG ACT CAG	15	458
90ml02	AGA AAT TTG ATG ACT C	16	459
90ml03	A AAT TTG ATG ACT ATG ACT	16	460
90ml04	AC CTG ATG ACT CAG	14	461
90ml05	AAT CTG ATG ACT CAG A	16	462
90ml06	AT CTG ATG ACT CAG ATG	16	463
90ml07	AT CTG ATG ACT CAG	14	464
90ml08	CTG ATG ACT CAG ATT G	16	465
90ml09	AGA AAT CTG ATG ACT C	16	466
90ml10	AGA AAT CTG ATG ACT	15	467

Table 3 cont

p90ml11	GA AGA AAT CTG ATG A	15	468
p90ml12	GGA AGA AAT CTG ATG A	16	469
p90ml13	GA AGA AAT CTG ATG AC	16	470
p90ml14	AGA AAT CTG ATG AC	14	471
p90wl15	AAT CTG TTA ACT CAG	15	472
p90wl16	T CTG TTA ACT CAG ATT	16	473
p90wl17	AT CTG TTA ACT CAG AG	15	474
p90wl18	AGA AAT TTG TTG ACT	16	475
p90wl19	GA AAT TTG TTG ACT C	15	476
p90wl20	AAT TTG TTG ACT CAG	15	477

CLAIMS

1. Method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising:

   a) if need be, releasing, isolating or concentrating the polynucleic acids present in the sample;

   b) if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair;

   c) hybridizing the polynucleic acids of step a) or b) with at least one of the following probes:

   probes specifically hybridizing to a target sequence comprising codon 30;

   probes specifically hybridizing to a target sequence comprising codon 46 and/or 48;

   probes specifically hybridizing to a target sequence comprising codon 50;

   probes specifically hybridizing to a target sequence comprising codon 54;

   probes specifically hybridizing to a target sequence comprising codon 82 and/or 84;

   probes specifically hybridizing to a target sequence comprising codon 90;

   or the complement of said probes;

   further characterized in that said probes specifically hybridize to any of the target sequences presented in figure 1, or to the complement of said target sequences;

   d) inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.

2. Method according to claim 1, further characterized in that said polynucleic acids of step a) or b) hybridize with at least two of the said probes, or to the complement of said probes.

3. Method according to claim 2, further characterized in that said probes are chosen from the following list: seq id no 7 to seq id no 477, or the complement of said probes.

4. Method according to any of claims 1 to 3, further characterized in that said primer pair consists of the primers with seq id no 3 and seq id no 4.

5. Method according to any of claims 1 to 3, further characterized in that:
step b) comprises amplifying a fragment of the protease gene with at least one 5'-primer specifically hybridizing to a target sequence located at position 210 to 260, in combination with at least one suitable 3'-primer, and
step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence or its complement, comprising codon 90.

6. Method according to any of claims 1 to 3, further characterized in that:
step b) comprises amplifying a fragment of the protease gene with at least one 3'-primer specifically hybridizing to a target sequence located at position 253 (codon 85) to position 300, in combination with at least one suitable 5'-primer, and
step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence or its complement, comprising any of codons 30, 46, 48, 50, 52, 54, 82 and 84.

7. Method according to claim 5, further characterized in that the 5'-primer is seq id no 5 and the 3'-primer is seq id no 4.

8. Method according to claim 6, further characterized in that the 5'-primer is seq id no 3 and the 3'-primer is seq id no 6.

9. A probe as defined in any of claims 1 to 3, for use in a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample.

10. A nucleic acid comprising a nucleotide sequence represented by any of the following SEQ ID numbers: SEQ ID NO 478, SEQ ID NO 479, SEQ ID NO 480, SEQ ID NO 481, SEQ ID NO 482, SEQ ID NO 483, SEQ ID NO 484, SEQ ID NO 485, SEQ ID NO 486, SEQ ID NO 487, SEQ ID NO 488, SEQ ID NO 489, SEQ ID NO 490, SEQ ID NO 491, SEQ ID NO 492, SEQ ID NO 493, SEQ ID NO 494, SEQ ID NO 495, SEQ ID NO 496, SEQ ID NO 497, SEQ ID NO 498, SEQ ID NO 499 and SEQ ID NO 500;

or a fragment thereof, wherein said fragment consists of at least two contiguous nucleotides and contains at least one polymorphic nucleotide.

11. A primer as defined in any of claims 4 to 8, for use in a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample.

12. A diagnostic kit enabling a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said kit comprising:

a) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in said sample;

b) when appropriate, at least one of the primers of any of claims 4 to 6;

c) at least one of the probes of any of claims 1 to 3, possibly fixed to a solid support;

d) a hybridization buffer, or components necessary for producing said buffer;

e) a wash solution, or components necessary for producing said solution;

f) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;

h) when appropriate, a means for attaching said probe to a solid support.

ABSTRACT

Method for detection of drug-selected mutations in the protease gene.

The present invention relates to a method for the rapid and reliable detection of drug-selected mutations in the HIV protease gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridisation assay.

More particularly, the present invention relates to a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising:

a) if need be, releasing, isolating or concentrating the polynucleic acids present in the sample;

b) if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair;

c) hybridizing the polynucleic acids of step a) or b) with at least one of the following probes:

probes specifically hybridizing to a target sequence comprising codon 30;

probes specifically hybridizing to a target sequence comprising codon 46 and/or 48;

probes specifically hybridizing to a target sequence comprising codon 50;

probes specifically hybridizing to a target sequence comprising codon 54;

probes specifically hybridizing to a target sequence comprising codon 82 and/or 84;

probes specifically hybridizing to a target sequence comprising codon 90;

or the complement of said probes;

further characterized in that said probes specifically hybridize to any of the target sequences presented in figure 1, or the complement of said target sequences;

d) inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.

1/21

Figure 1

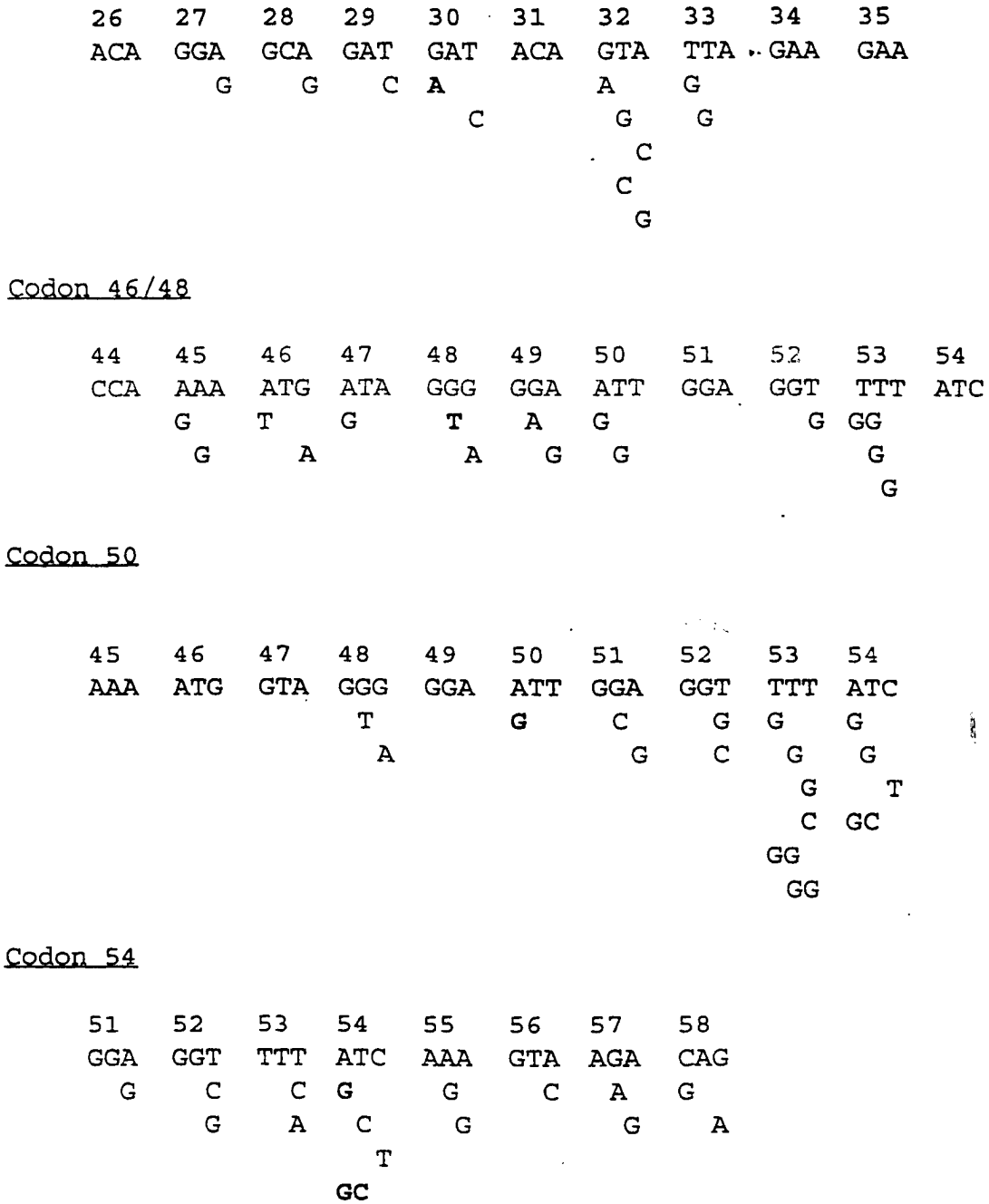

Codon 30
26 27 28 29 30 31 32 33 34 35
ACA GGA GCA GAT GAT ACA GTA TTA GAA GAA
G G C A A G
C G G
C
C
G
Codon 46/48
44 45 46 47 48 49 50 51 52 53 54
CCA AAA ATG ATA GGG GGA ATT GGA GGT TTT ATC
G T G T A G G G GG
G A A G G G
G
Codon 50
45 46 47 48 49 50 51 52 53 54
AAA ATG GTA GGG GGA ATT GGA GGT TTT ATC
T G C G G G G
A G C G G T
C GC
GG
GG
Codon 54
51 52 53 54 55 56 57 58
GGA GGT TTT ATC AAA GTA AGA CAG
G C C G G C A G
G A C G G A
T
GC

Figure 1 cont'd

Codon 82/84

78	79	80	81	82	83	84	85	86	87
GGA	CCT	ACA	CCT	GTC	AAC	ATA	ATT	GGA	AGA
	A	T	G	**T**	C	G	G		
	G	T	C	**A**	T		G		
		G	A	**C**			GG		
				T			C		
				AC					
				TC					

Codon 90

86	87	88	89	90	91	92	93	94
GGA	AGA	AAT	CTG	TTG	ACT	CAG	ATT	GGT
	C	C	A	**A**	C	A	C	G
	A	C	T	C	A	A	G	C
	G		C	A			G	A
			A	AA			A	
			A A				GG	
							C G	

3/24

FIGURE 2 A

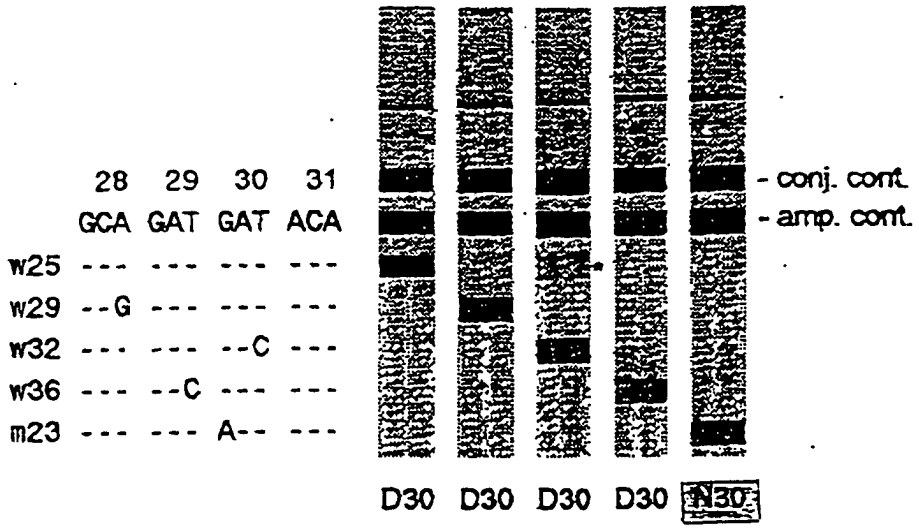


FIGURE 2 B

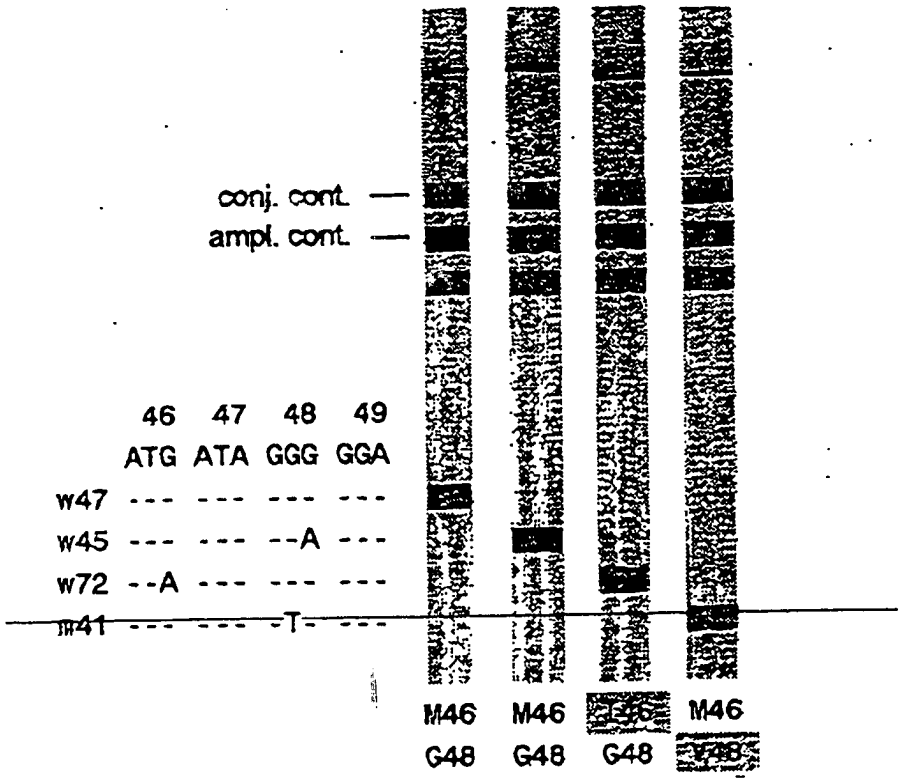
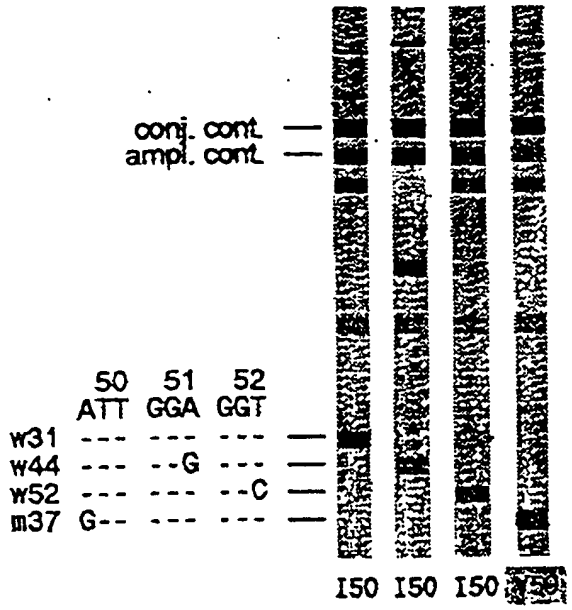
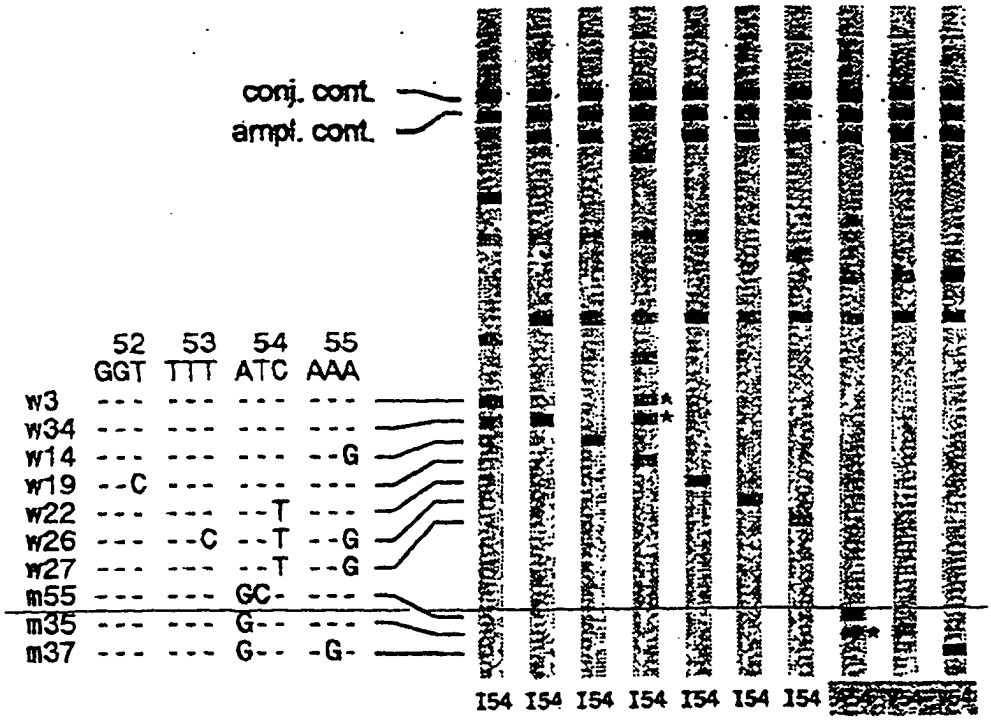

conj. cont.
ampl. cont.
46 47 48 49
ATG ATA GGG GGA
w47 --- --- --- ---
w45 --- --- --A ---
w72 --A --- --- ---
w41 --- --- -T- ---
M46 M46 M46 M46
G48 G48 G48 V48

FIGURE 2 C


conj. cont.
ampl. cont.
50 51 52
ATT GGA GGT
w31 --- --- ---
w44 --- --G ---
w52 --- --- --C
m37 G-- --- ---
I50 I50 I50 I50

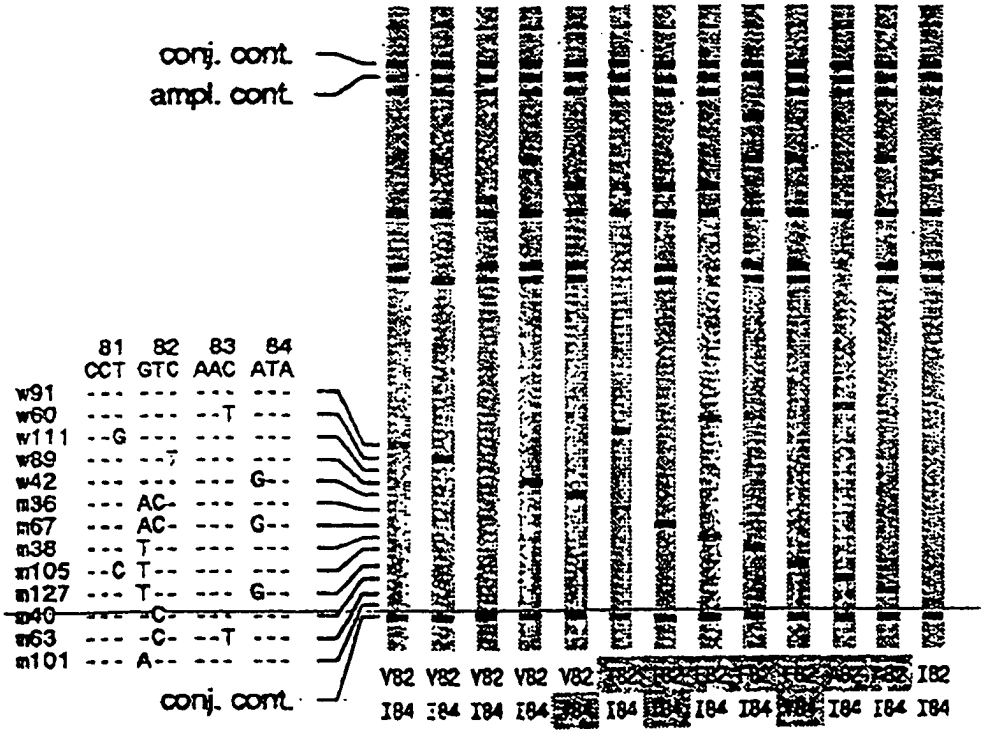
6/27

FIGURE 2 D



7/21

FIGURE 2 E



8/24

FIGURE 2 F

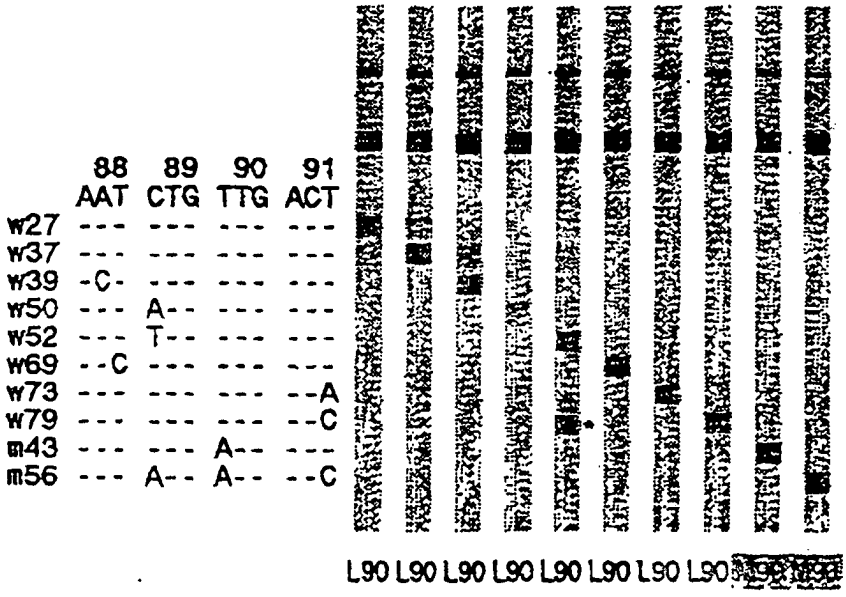


FIGURE 3

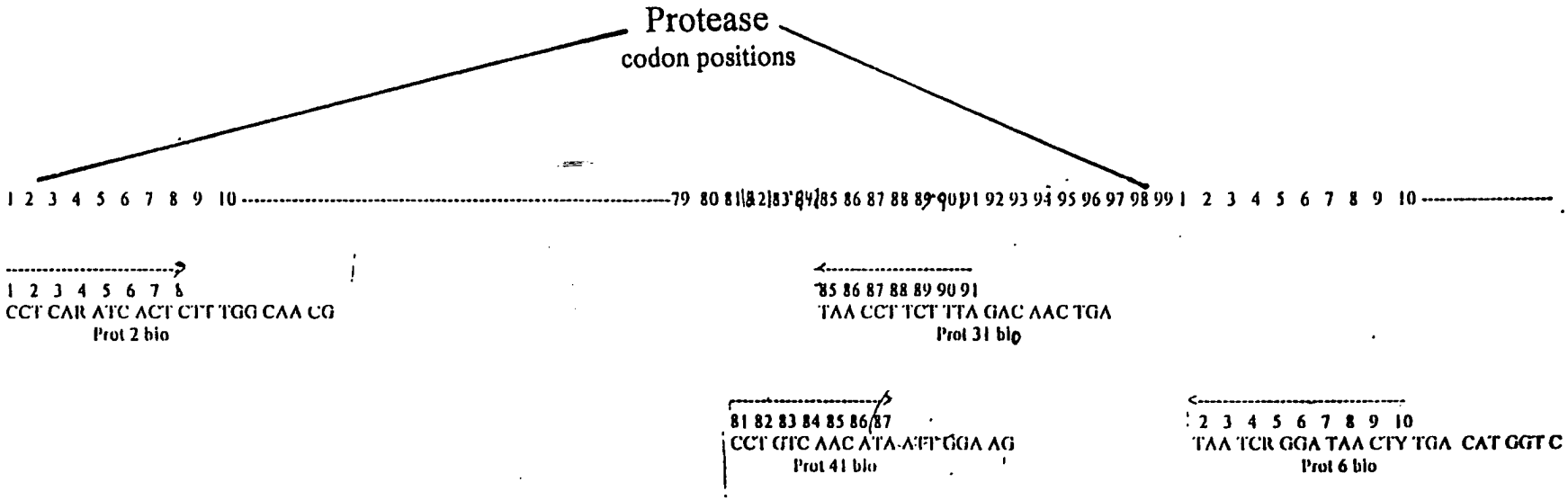

Protease
codon positions
1 2 3 4 5 6 7 8 9 10 ---- 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8
CCT CAR ATC ACT CTT TGG CAA CG
Prot 2 bio
85 86 87 88 89 90 91
TAA CCT TCT TTA GAC AAC TGA
Prot 31 bio
81 82 83 84 85 86 87
CCT GTC AAC ATA-ATT GGA AG
Prot 41 bio
2 3 4 5 6 7 8 9 10
TAA TCR GGA TAA CTY TGA CAT GGT C
Prot 6 bio
Primer positions

FIGURE 4 A

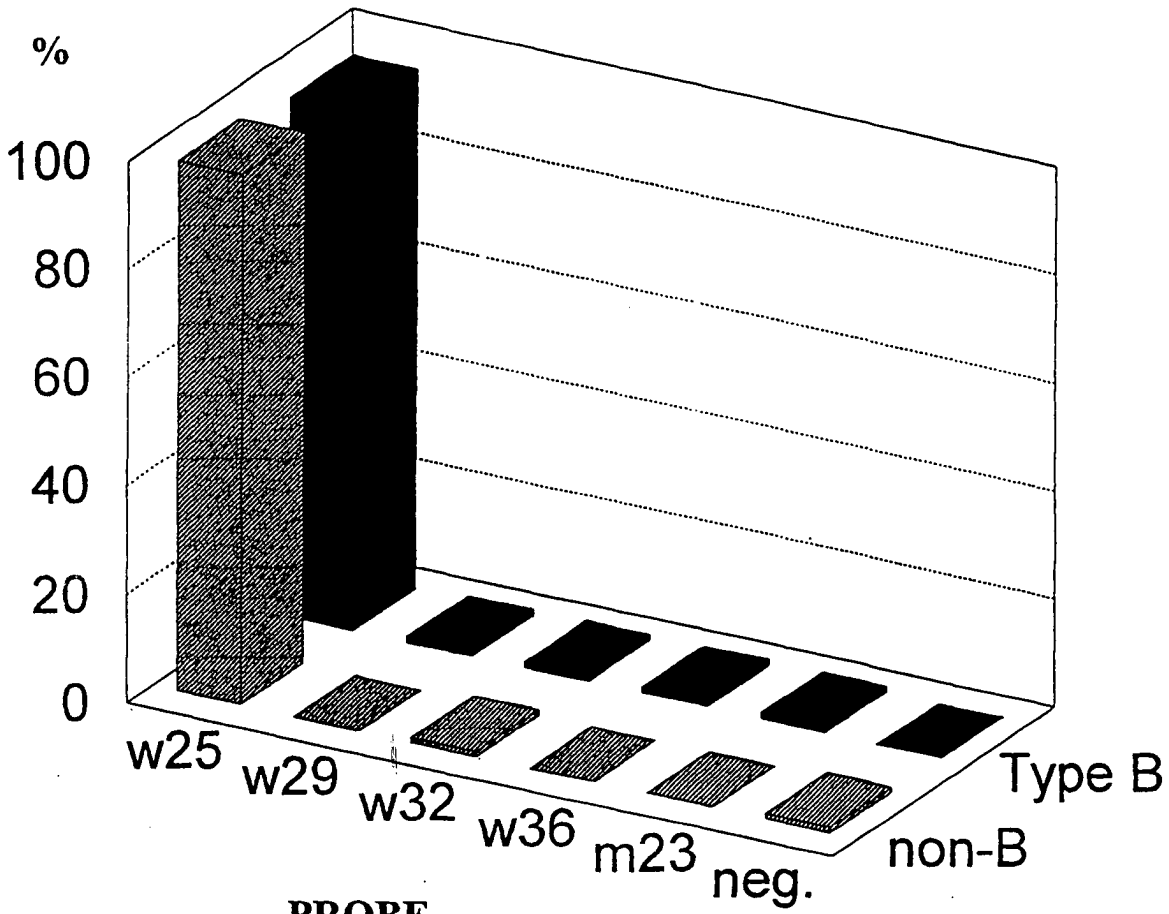


FIGURE 4 B

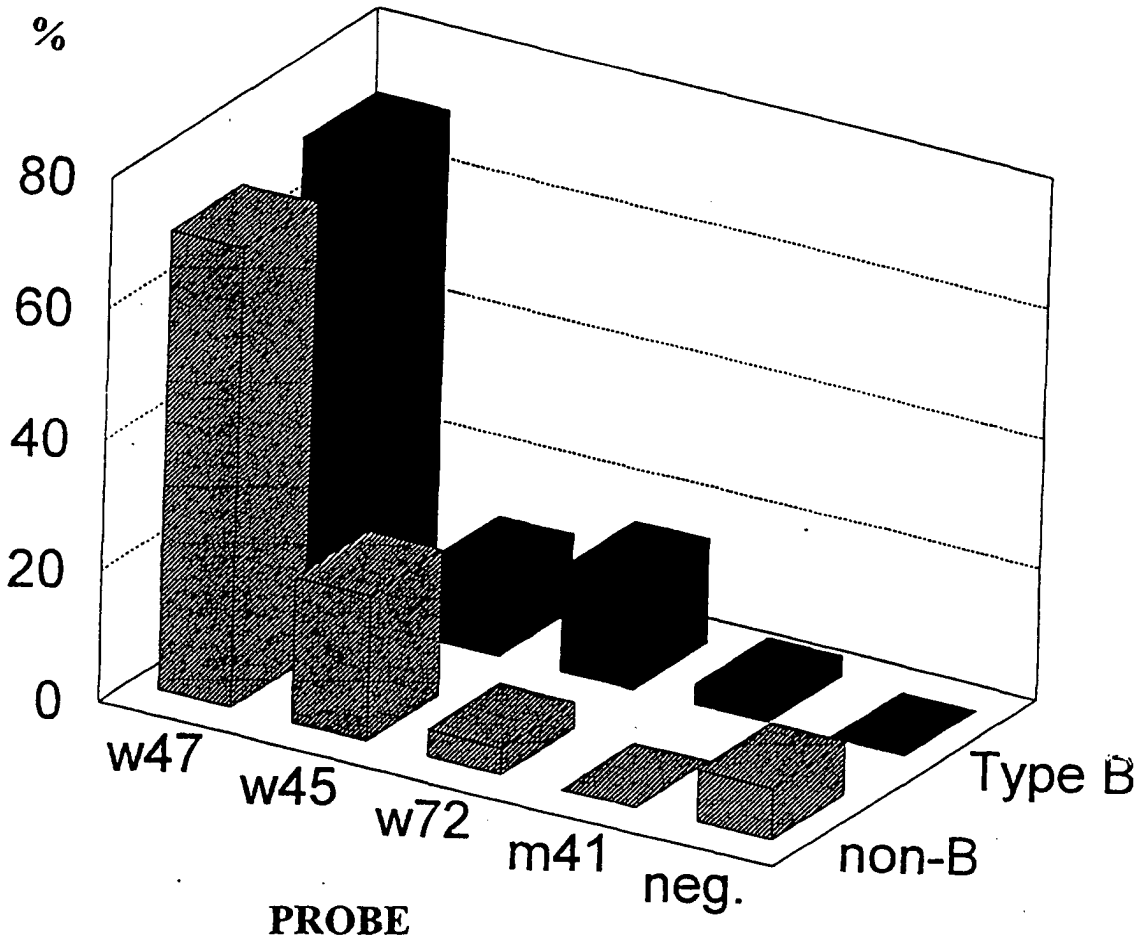


FIGURE 4 C

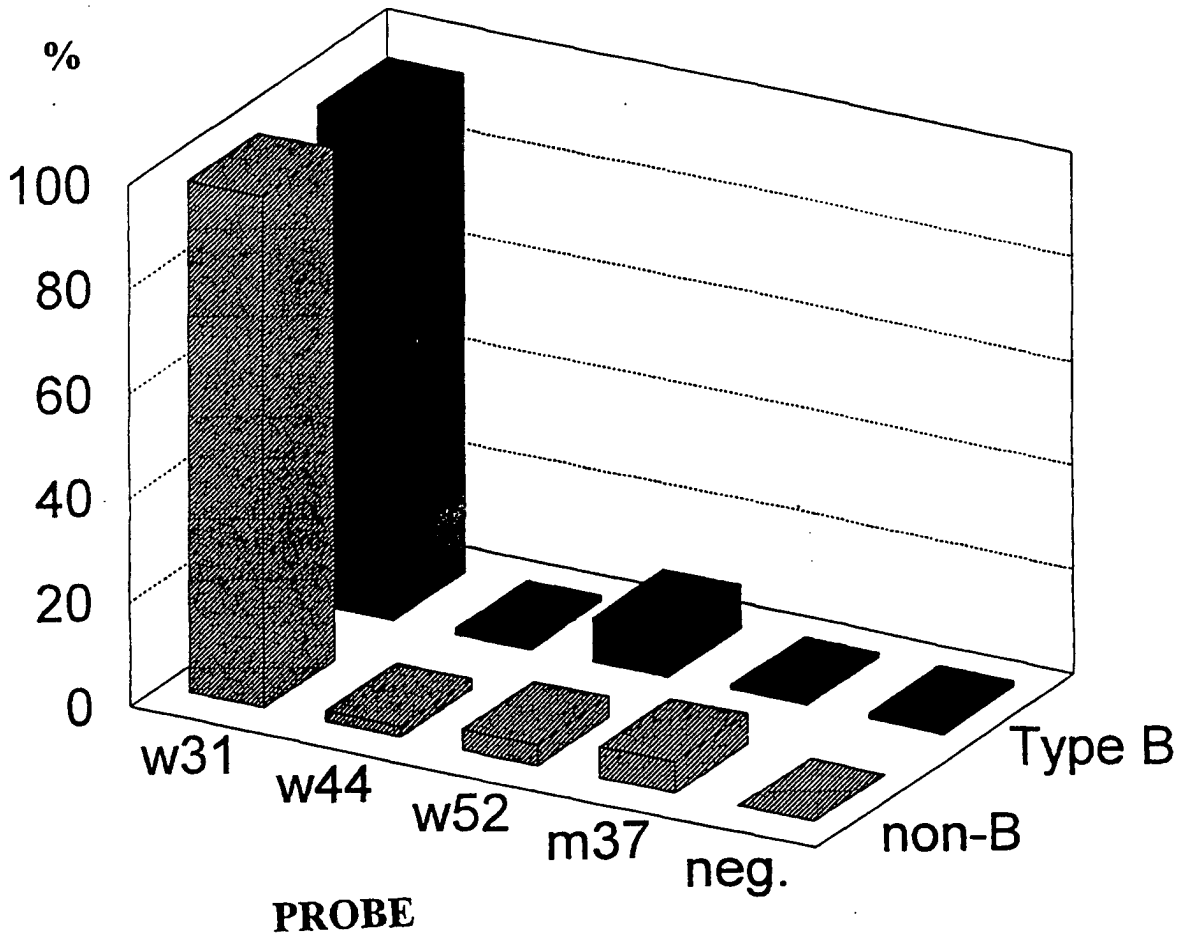
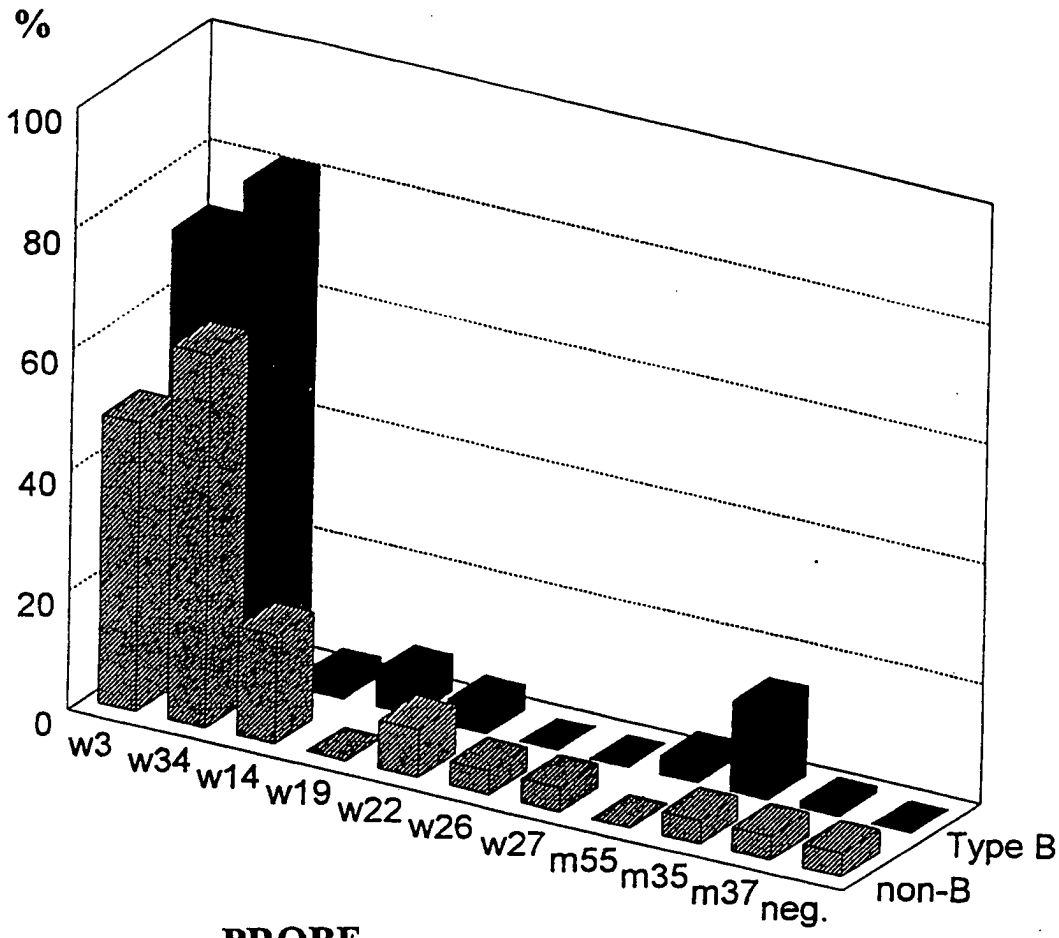
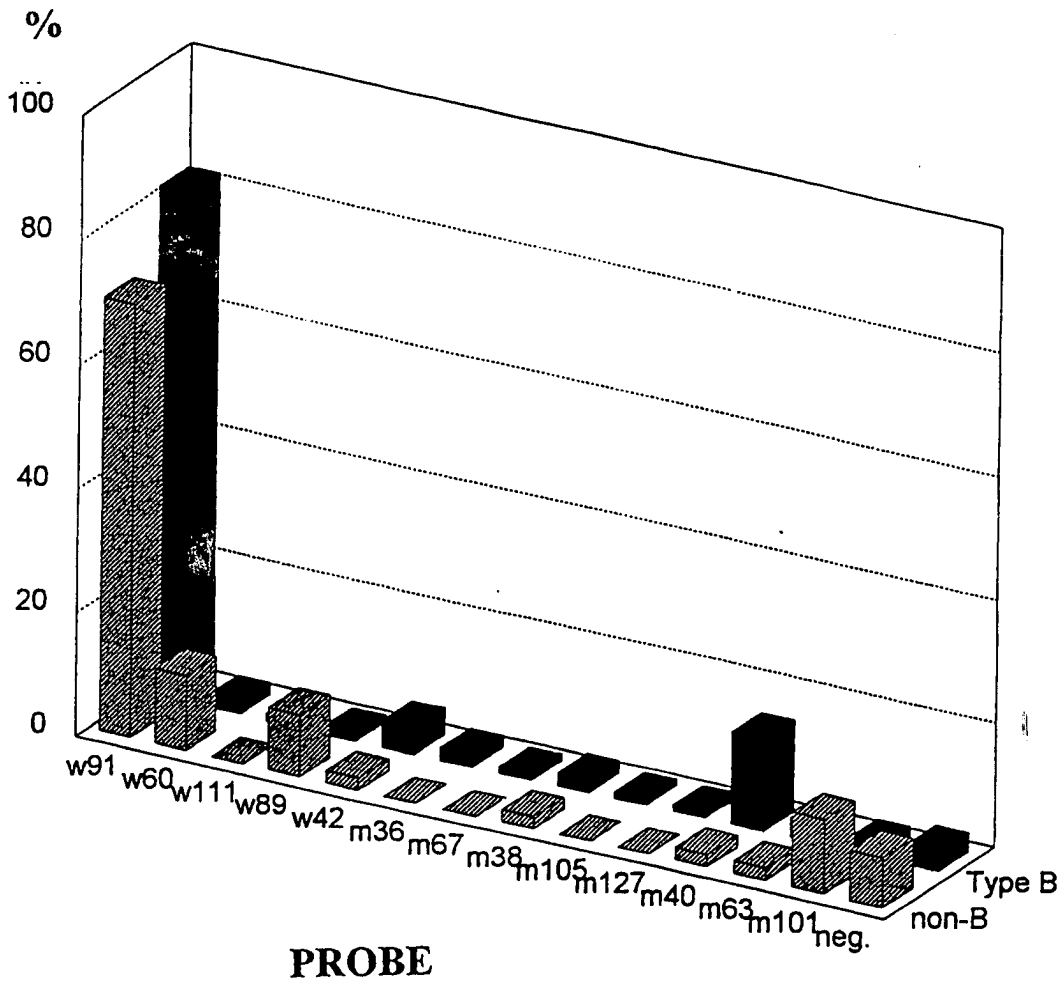


FIGURE 4 D


%
100
80
60
40
20
0
w3
w34
w14
w19
w22
w26
w27
m55
m35
m37
neg.
Type B
non-B
PROBE

74/91

FIGURE 4 E



PROBE

75/91

FIGURE 4 F

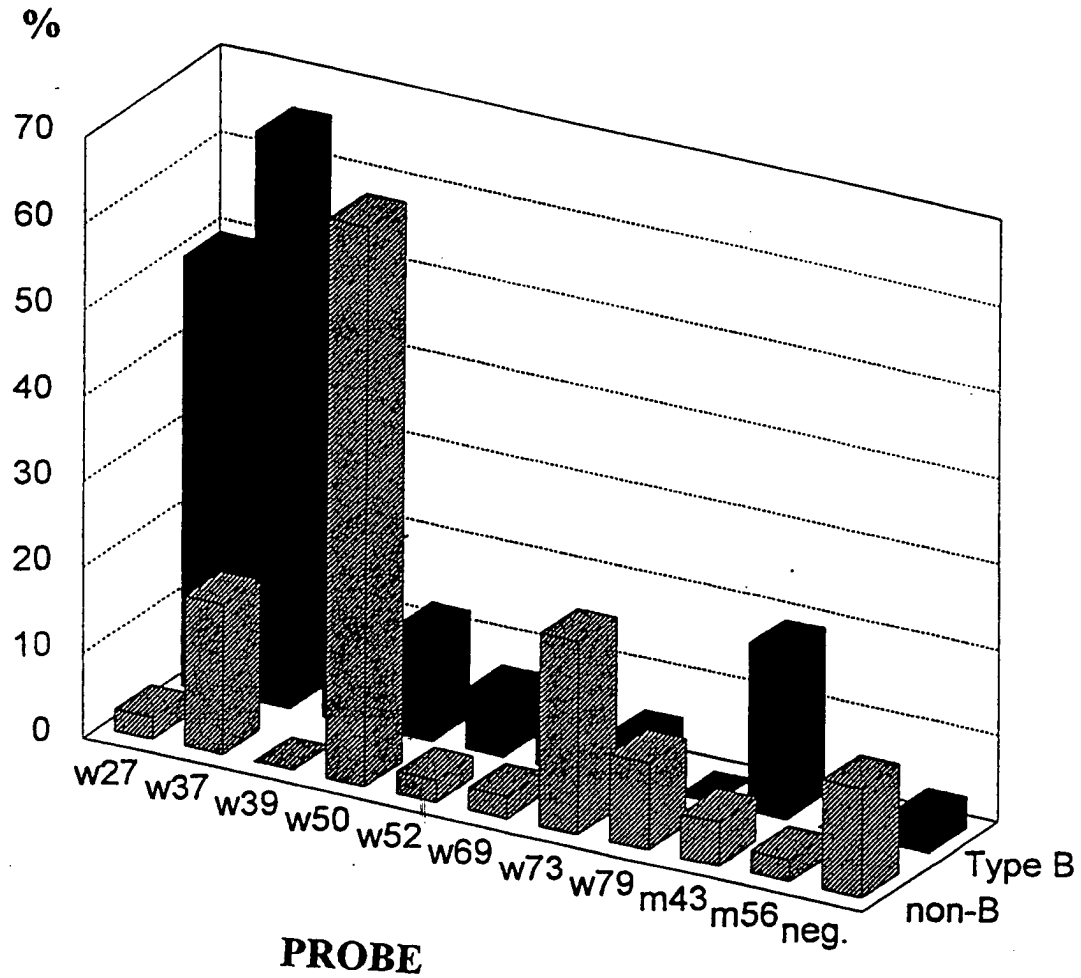
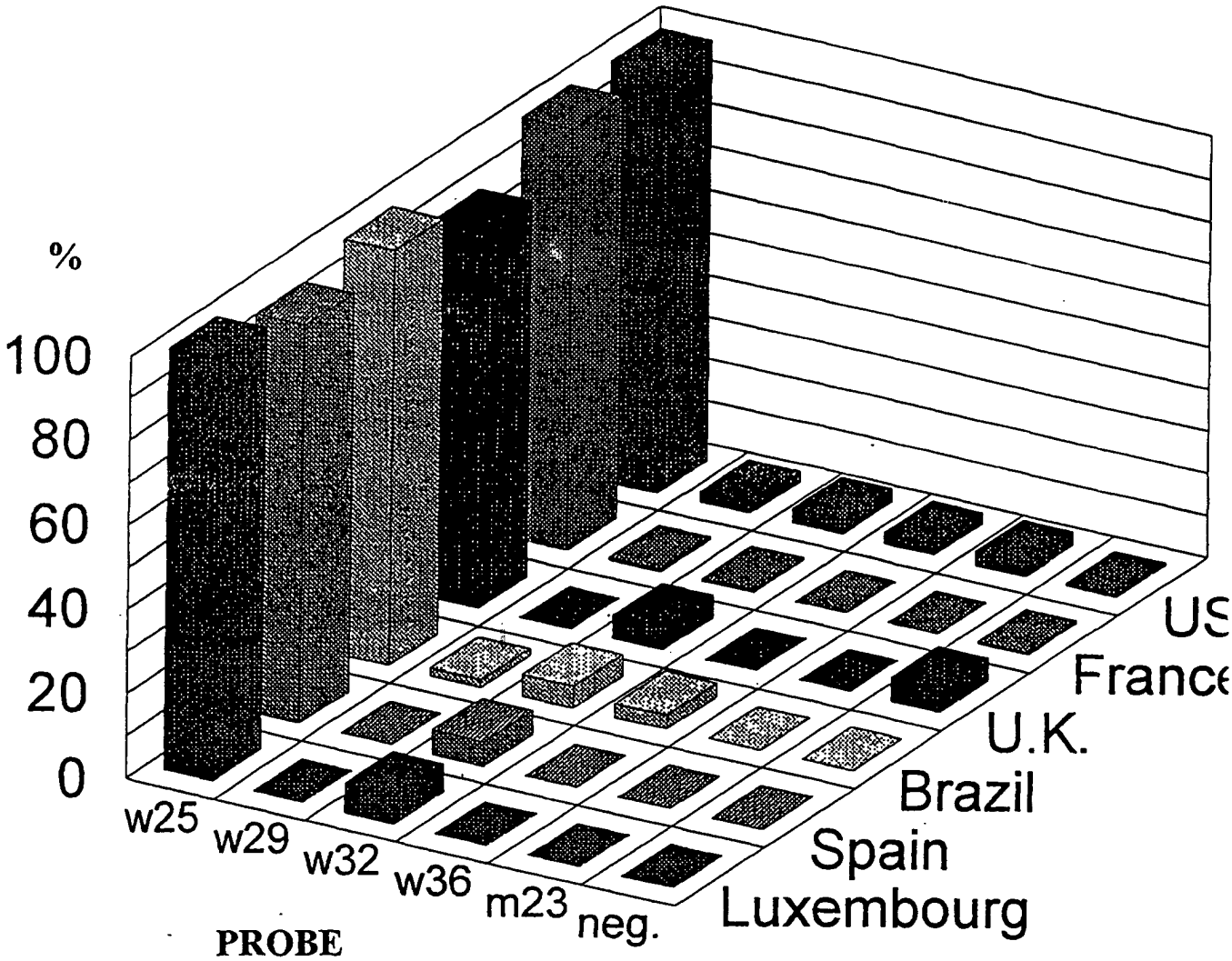


FIGURE 5 A


%
100
80
60
40
20
0
w25
w29
w32
w36
m23
neg.
PROBE
Luxembourg
Spain
Brazil
U.K.
France
US

17/27

FIGURE 5 B

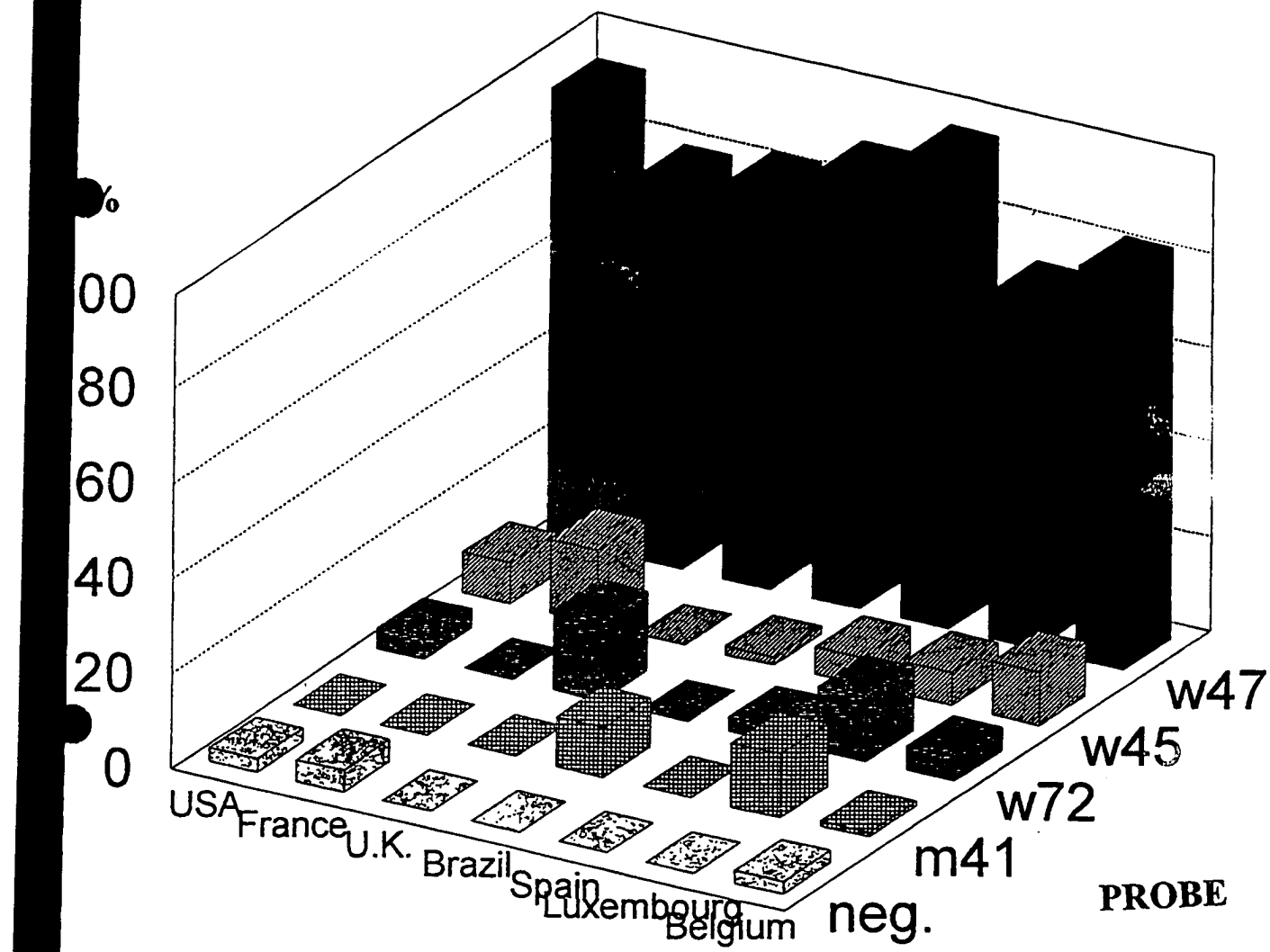
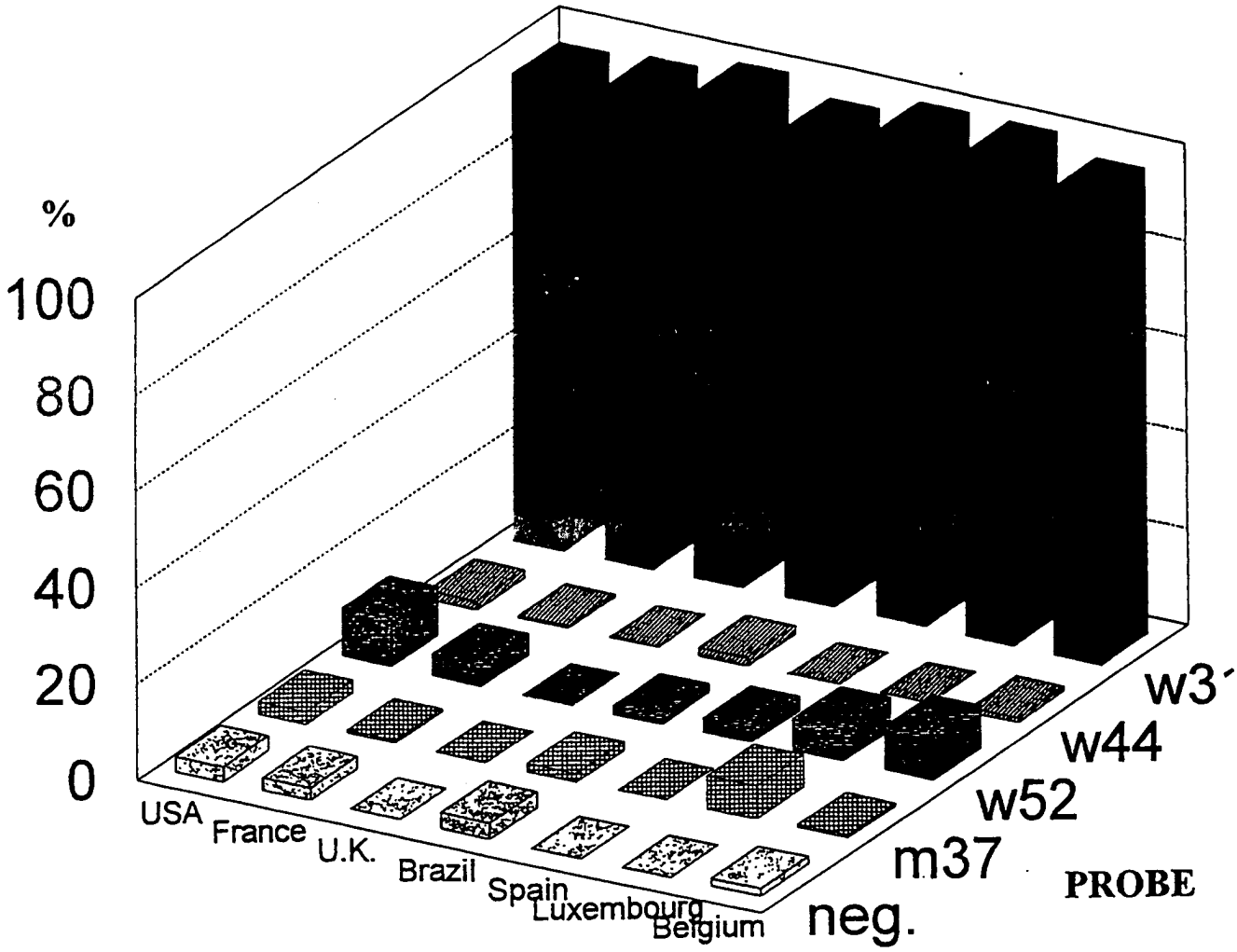


FIGURE 5 C


%
100
80
60
40
20
0
USA
France
U.K.
Brazil
Spain
Luxembourg
Belgium
neg.
m37
w52
w44
w3
PROBE

79/91

FIGURE 5 D

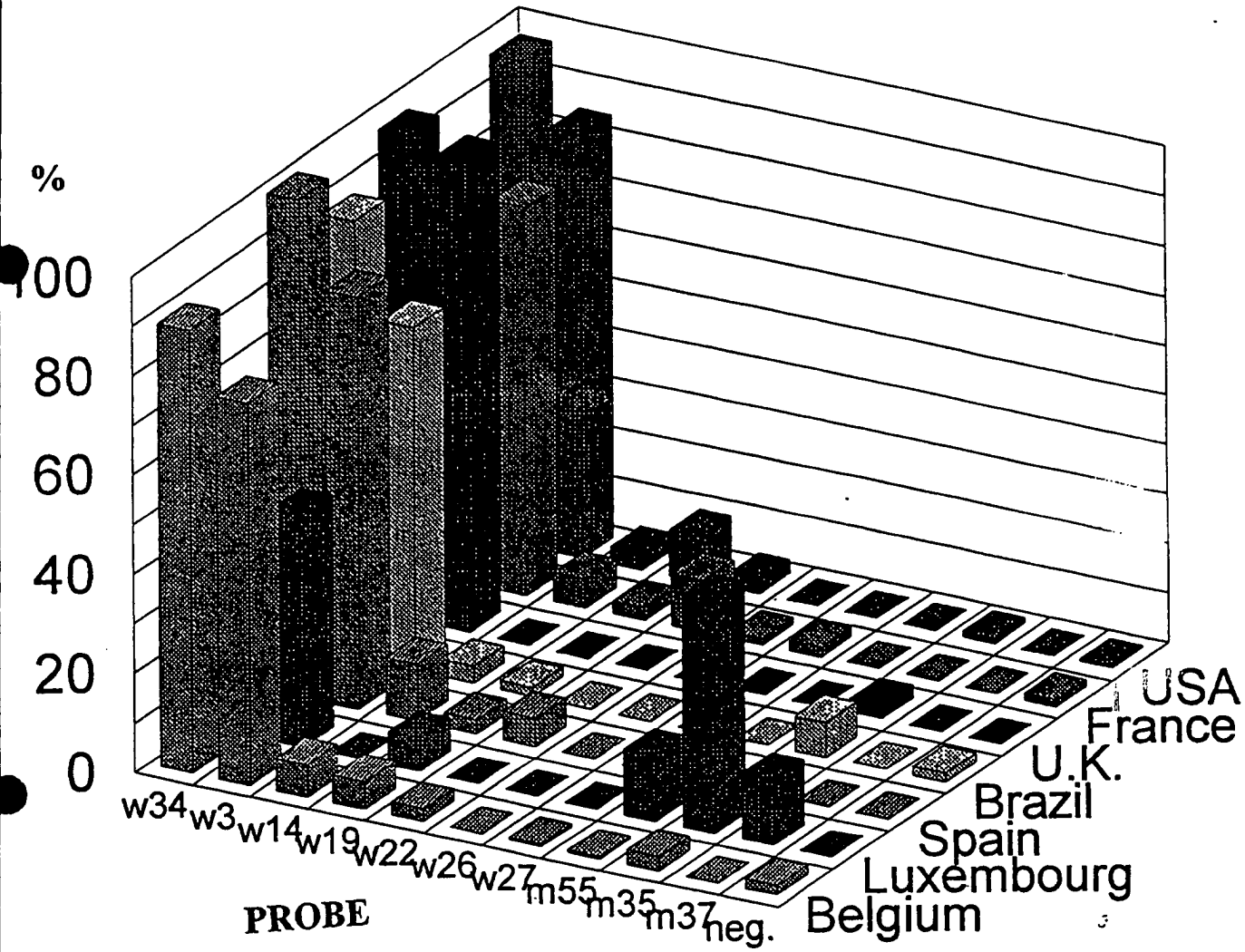
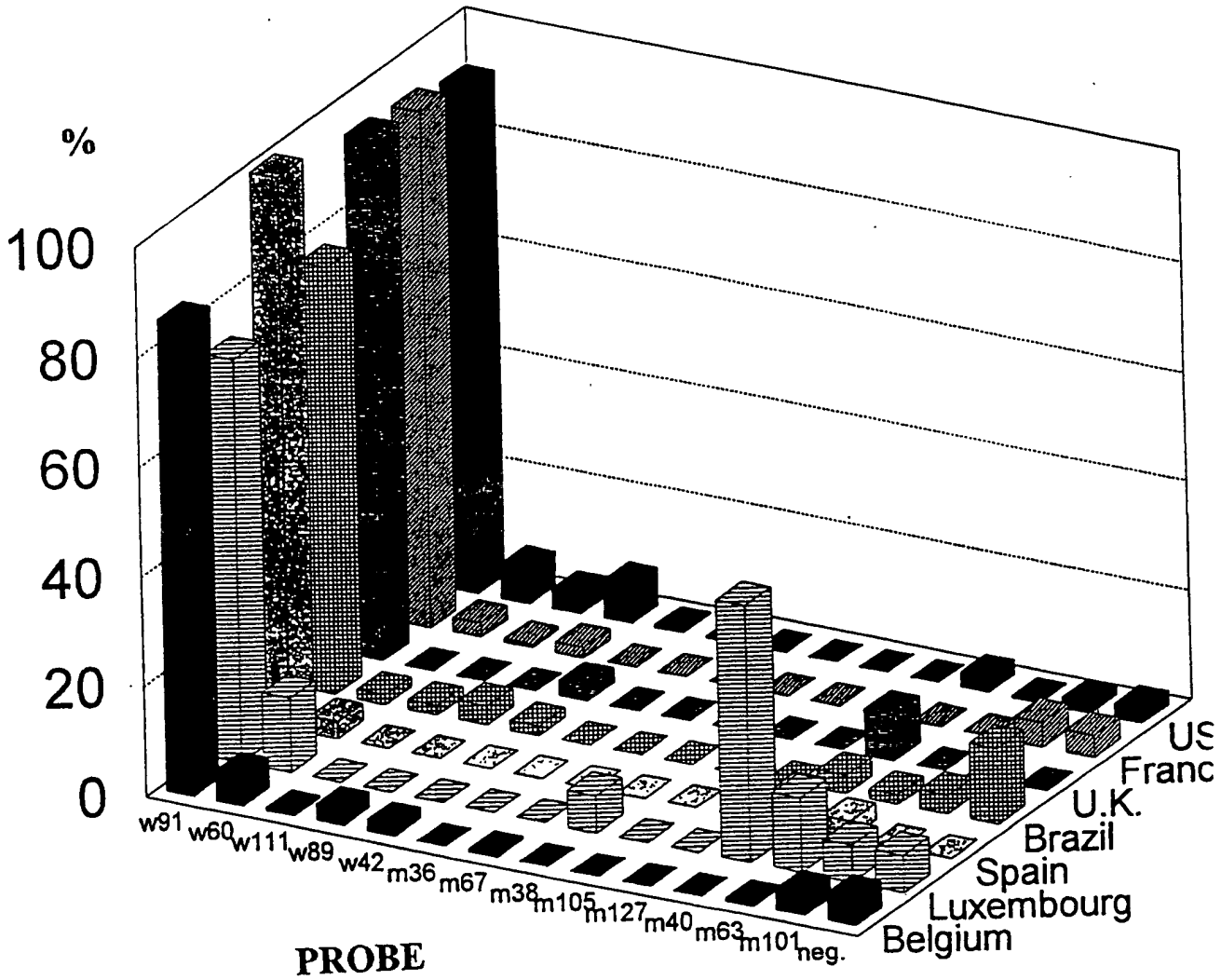
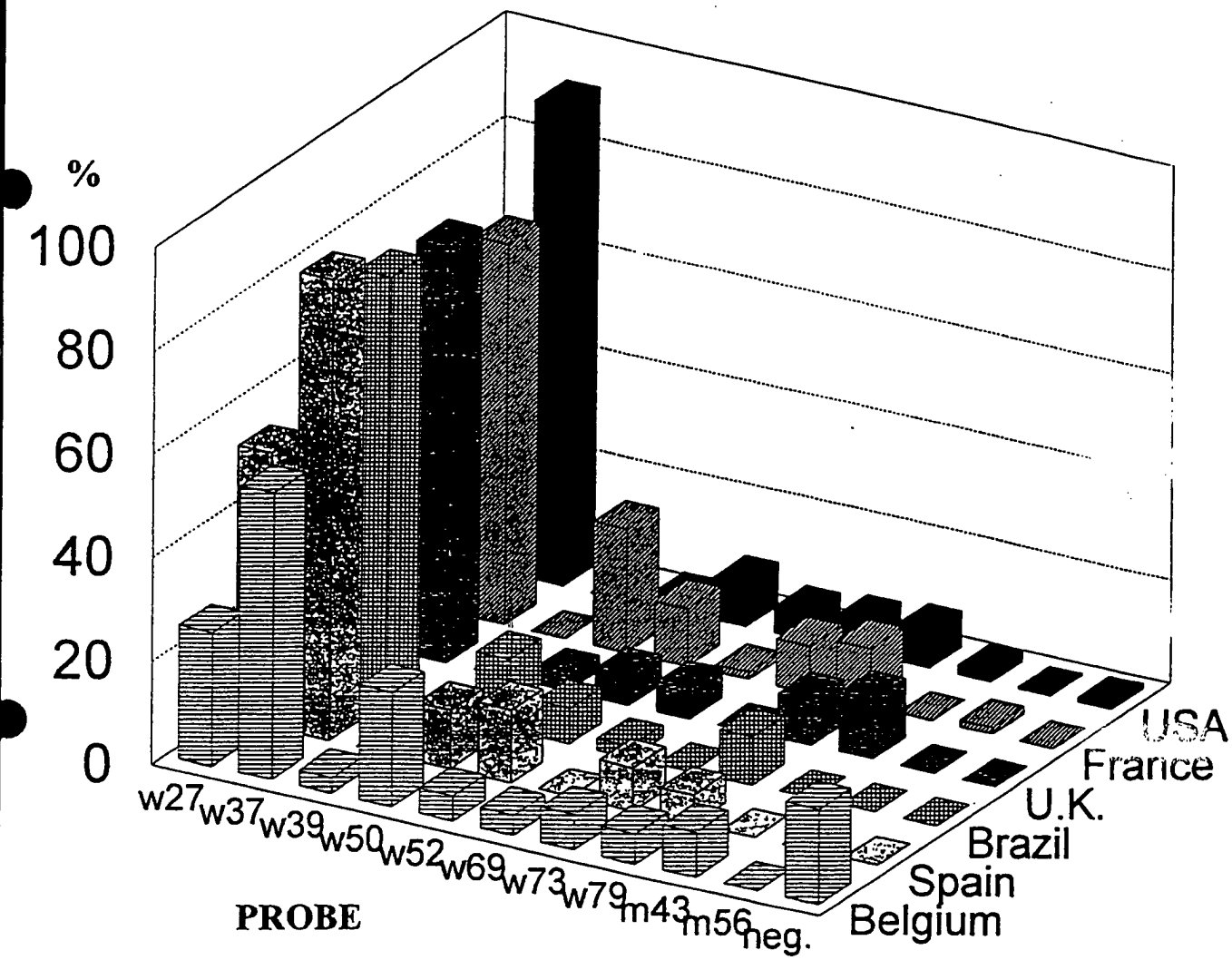


FIGURE 5 E



PROBE

FIGURE 5 F


%
100
80
60
40
20
0
w27
w37
w39
w50
w52
w69
w73
w79
m43
m56
neg.
PROBE
Belgium
Spain
Brazil
U.K.
France
USA